2005

Resident enteric flora modulates the development of colitis in gnotobiotic mice

Albert E. Jergens
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Resident enteric flora modulates the development of colitis in gnotobiotic mice

by

Albert E. Jergens

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

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Iowa State University

Ames, Iowa

2005

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Signature was redacted for privacy.

For the Major Program
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CHAPTER 1. GENERAL INTRODUCTION

Research Problem: Chronic intestinal inflammation, as seen with inflammatory bowel disease in humans, results from aberrant and poorly understood mucosal immune responses to intraluminal bacteria of the gastrointestinal tract in genetically susceptible individuals. Although considerable research evidence indicates that luminal bacteria provide the antigenic stimulation for intestinal inflammation, the evaluation of bacterial-specific host responses mediating intestinal injury (as a consequence of changes in the composition of the resident enteric flora) have not been performed.

Overview of Inflammatory Bowel Disease in Humans:

Introduction

The chronic idiopathic inflammatory bowel diseases (IBD), which include Crohn’s disease (CD), and ulcerative colitis (UC), are recognized as important causes of gastrointestinal disease in adults and children. Inflammatory bowel disease occurs worldwide, although it is more common in some regions (e.g., United States, United Kingdom, and Scandinavia) than in others with incidence rates of 4-10/100,000 persons per year and prevalence rates between 40-100/100,000 persons.(1) These disorders are most commonly diagnosed between the third and fourth decades of life, with no difference noted between males and females. Approximately 20% of all patients with IBD develop symptoms during childhood, with about 5% being diagnosed before 10 years of age. The ratio of UC to CD varies widely according to the country of origin of the survey but in most countries UC is found with greater frequency than CD.(2)
Defining Ulcerative Colitis and Crohn’s Disease

Ulcerative Colitis

Ulcerative colitis is a disease in which the inflammatory response and histological changes are localized to the colon. The rectum is affected in 95% of patients with variable degrees of proximal extension. Inflammation is limited primarily to the mucosa and consists of diffuse involvement of variable severity with ulceration, edema, and hemorrhage predominating along the length of the colon. The characteristic histologic findings are lamina propria infiltration with mononuclear cells and neutrophils, crypt abcessation, distortion of the mucosal glands, and goblet cell depletion.

Crohn’s Disease

In contrast to UC, CD can involve any part of the gastrointestinal tract but also primarily involves the colon. Diseased segments frequently are separated by intervening normal bowel (e.g., patchy lesion distribution) with severe inflammation typically occurring as a transmural lesion. Histologic findings include small ulcerations, focal chronic inflammation extending down to the submucosa, and sometimes granuloma formation. The most common location for lesions is the ileocecal region, followed by the terminal ileum alone, diffuse small bowel involvement or focal colonic disease in decreasing order of frequency (e.g., ileocecal region>terminal ileum>small intestine.colon).

Intestinal Manifestations of Disease

Ulcerative Colitis

The most consistent feature of UC is the presence of blood and mucus in the stool, accompanied by lower abdominal cramping. The presence of diarrhea with blood and mucous as opposed to the absence to blood is used clinically to differentiate UC from
irritable bowel syndrome, an important and clinically-relevant IBD mimic. Ulcerative colitis is usually diagnosed earlier after the onset of symptoms than is CD because of the presence of blood in the stool, which generally alerts the person to the presence of a primary gastrointestinal disorder. Abdominal pain (cramping) is often present in the left lower quadrant with distal disease, and this discomfort may extend to involve the entire abdomen with pancolitis. Pediatric patients tend to have a higher frequency of pancolitis, a higher likelihood of proximal extension of the disease over time, and a higher risk of surgical intervention (e.g., colectomy) compared to adult-onset patients.(3)

**Crohn’s Disease**

In contrast to UC, the presentation of CD is often subtle leading to a delay in definitive diagnosis. Gastrointestinal symptoms depend upon the location, extent, and severity of inflammation. Gastroduodenal CD presents with early nausea, vomiting, epigastric pain, and/or dysphagia. Due to post-prandial pain and a delay in gastric emptying, patients with gastroduodenal CD often limit their caloric intake to diminish their discomfort. Colonic CD may mimic UC in that patients may present with diarrhea containing blood and mucous, lower abdominal cramping, and pain on defecation. Perianal disease is common, as are anal fissures and fistulae. Increasing abdominal cramping, distension, and emesis accompanied by borborygmi (e.g., intestinal “gurgling” are signs of progression of the inflammatory process to focal luminal stenosis causing partial/complete obstruction.

The course of IBD may be complicated by one or more extraintestinal manifestations including fever, weight loss, delayed growth and sexual maturation in children, arthralgia/arthritis, mucocutaneous lesions, ophthalmologic complications, hepatobiliary disease, and/or renal disease.(4) These extraintestinal lesions may be diagnosed concurrently
with active bowel disease, following a flare of IBD, or occasionally, may be the initial presentation of IBD preceding intestinal symptomatology.

**Diagnosis of IBD**

The importance of excluding enteric pathogens before confirming a diagnosis of IBD, or during IBD exacerbations, cannot be overemphasized. There are a diverse number of bacterial pathogens that may mimic IBD and are discussed in a separate section of this dissertation. Once enteric infections are excluded, further diagnostic evaluation, including endoscopic mucosal biopsy which is required for definitive diagnosis, is performed (Table 1).

**Table 1. Tests used to diagnose IBD**

<table>
<thead>
<tr>
<th>Test</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete blood counts</td>
<td>Microcytic anemia, leukocytosis with band forms, thrombocytosis</td>
</tr>
<tr>
<td>Acute-phase reactants</td>
<td>Elevated sedimentation rate and serum orosomucoid, and C-reactive protein levels</td>
</tr>
<tr>
<td>Chemistries</td>
<td>Low serum iron level, hypoalbuminemia, elevated liver enzyme levels</td>
</tr>
<tr>
<td>Special serologic tests</td>
<td>pANCA, ASCA</td>
</tr>
<tr>
<td>Stool examinations</td>
<td>Exclude bacterial pathogens, ova and parasites, occult blood, and fecal leukocytes</td>
</tr>
<tr>
<td>Endoscopic evaluation</td>
<td>Esophagogastroduodenoscopy with biopsy, colonoscopy with biopsy</td>
</tr>
<tr>
<td>Radiologic evaluation</td>
<td>Upper gastrointestinal tract with small bowel follow-through, enteroclysis (as indicated), barium enema</td>
</tr>
</tbody>
</table>

Table 1. Diagnostic tests for IBD. These tests are generally performed in a step-wise manner but always require histopathologic evaluation of intestinal biopsy specimens for diagnosis. pANCA= perinuclear anti-neutrophil cytoplasmic antibody; ASCA= anti-*Saccharomyces cerevisiae* antibodies. Modified from Hendrickson BA, Gokhale R, Cho J. Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin Micro Rev* 2002; 15:79-94.

**Treatment of IBD**

The management of IBD involves several strategies including: (1) control of acute symptoms (e.g., diarrhea, obstruction, megacolon), (2) induction and maintenance of remission (generally with corticosteroids, 5-ASA derivatives [sulfasalazine], antibiotics
[metronidazole, ciprofloxacin], immunosuppressive drugs [azathioprine, cyclosporine], and biologic therapies [infliximab → a novel TNF-α monoclonal antibody], (3) surgical intervention for masses and stenoses, (4) nutritional support [dietary modification, use of pre/probiotics] and (5) cancer surveillance.

**Prognosis of UC and CD**

The long-term prognosis for patients with IBD is extremely variable and is a consequence of differences in disease extent, frequency, and severity of disease. For UC, the relapse rate is approximately 40% during the first year following intensive medical therapy with an additional 13% and 16% during the second and third years, respectively.(5) Most of these relapses may be controlled with medical therapy, but surgery will be required in approximately 1/3 of patients necessitating intensive treatment for complications (e.g., toxic dilatation of the colon).(6,7) For CD, the need for surgery occurs in greater than 50% of patients and a majority of patients will experience recurrent disease with the need for subsequent operative intervention.(8) The prognosis of IBD must also take into account the increased risk of colon carcinoma that is present in patients who have pan-UC for more than 8 years (9), but which may also occur in patients who have CD for more than 15 years.(10) As a result, it is recommended that high risk patients who have IBD undergo endoscopic surveillance with multiple colonic biopsies as part of the long-term management of their disease.

In summary, idiopathic IBD represents two distinct entities that share a number of clinical similarities and disease manifestations. Although the exact cause for UC and CD remains unknown, it is reasonable to expect that the further development and use of animal models to explore mechanisms of gastrointestinal inflammation will be important.
Additionally, animal models will continue to provide important insights into the cause for IBD in humans, as well as aid in the design of more effective treatment modalities.

**ETIOPATHOGENESIS OF INFLAMMATORY BOWEL DISEASE: MICROBIAL INFLUENCES IN ANIMAL MODELS OF DISEASE**

**Hypotheses Regarding Etiopathogenesis**

Human inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are poorly understood chronic immunologically-mediated diseases of unknown etiology. Although numerous questions regarding the cause for IBD remain, familial and epidemiologic data indicate that these debilitating intestinal inflammatory disorders result from complex interactions between a genetically susceptible host, the mucosal immune system, and environmental factors. (Figure 1)

![Figure 1. Host-microbial-environmental interactions which contribute to the development of IBD. Note that microbial factors consist of adjuvants and antigens derived from commensal enteric bacteria. With a loss of tolerance, the scale is tipped toward mucosal inflammation which may culminate in IBD.](image-url)
However, neither the specific genes or environmental triggers have been conclusively identified. Although the preponderance of data indicates that commensal luminal bacterial constituents provide the constant antigenic drive for chronic intestinal inflammation, other possibilities include dietary contributions, persistent pathogens, and alterations of the virulence factors of commensal bacteria. This section will briefly discuss the most widely accepted etiologic hypotheses for IBD (Table 2).

### Table 2. Etiologic theories for IBD

<table>
<thead>
<tr>
<th>Persistent pathogen: Mycobacterium paratuberculosis, virulent E. coli, Pseudomonas species, Listeria monocytogenes, Helicobacter species, measles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defective clearance of pathogenic and commensal bacteria</td>
</tr>
<tr>
<td>Defective mucosal barrier: enhanced permeability, ineffective healing</td>
</tr>
<tr>
<td>Dysbiosis: altered balance of protective/detrimental commensals</td>
</tr>
<tr>
<td>Dysregulated immune response: overly aggressive effectors, loss of tolerance</td>
</tr>
</tbody>
</table>


**Persistent Pathogenic Infection**

A number of microbial pathogens have been proposed as causes of UC or CD, including *Mycobacterium paratuberculosis*, adherent/invasive *E. coli*, *Pseudomonas* spp., *Listeria monocytogenes*, or *Helicobacter* species. To date, the vast majority of these organisms have not been confirmed by blinded, controlled investigations. Adherent/invasive *E. coli* may contribute to post-operative recurrence of Crohn's disease and the role of
*Helicobacter* species has yet to be fully explored. The availability of sensitive molecular techniques to detect microbial agents may yet allow identification of a specific bacterial pathogen.

**Impaired Clearance of Pathogenic and Commensal Organisms**

Defective killing of invading commensal bacteria, opportunistic bacteria, or traditional enteric pathogens may cause tissue inflammation and the induction of pathogenic immune responses. Some humans with CD have defective neutrophil function.\(^\text{14}\) It has been shown that some *Bacteroides* species impair neutrophil phagocytosis and microbicidal activity, suggesting that enteric bacteria mediate these defects. It is noteworthy that microbial products can also inhibit host defenses. *Bacteroides* species may decrease neutrophil phagocytosis and bacterial killing \(^\text{15}\) and defective T-cell function may foster the growth of opportunistic pathogens, such as intestinal *Helicobacters* which cause typhlitis and hepatobiliary disease in T-cell deficient mice.\(^\text{16}\) Lastly, suppression of neutrophil function by nicotine and the administration of non-steroidal anti-inflammatory drugs (NSAIDs) could explain several other environmental risk factors for CD.

**Defective Mucosal Barrier Function**

Intrinsic (genetically determined) or acquired (environmental agent) defects in mucosal barrier function or healing can lead to continuous uptake of luminal antigens that overwhelm host protective mechanisms. Endogenous luminal bacteria and their phlogistic products traverse the inflamed mucosa in IBD, especially in CD and experimental enterocolitis.\(^\text{17}\) A correlation between disease activity and altered mucosal permeability in both CD and UC, and enhanced permeability predicting flares of CD, has been previously reported.\(^\text{18-20}\) Additionally, increased uptake of bacterial products derived from
translocating bacteria suggests an intrinsic defect in mucosal barrier function which may precipitate intestinal inflammation. Previous studies have shown that mucosally-associated bacterial concentrations are dramatically increased in IBD patients. (21)

**Altered Balance of Protective/Detrimental Commensal Bacteria**

Normal (otherwise healthy) hosts may develop chronic intestinal inflammation as a consequence of altered composition of commensal enteric bacteria. The balance between beneficial and detrimental enteric bacteria appears to be disturbed in patients with IBD, especially in the western, industrialized populations where IBD is most prevalent. In brief, increased fecal concentrations of intestinal bacteria (predominantly anaerobes) are present in CD patients and their asymptomatic relatives. (22) Similar observations in experimental models have demonstrated the inflammatory potential of bacterial species, providing additional support for the concept that composition of the resident microflora modulates disease severity. Similar to human IBD, streptococci, clostridial species, and *Bacteroides* (particularly *B. vulgatus*) may cause colitis in specific animal models. In contrast, lactobacilli and bifidobacteria have protective roles in pro-biotic and pre-biotic studies.

**Disregulated Immune Responses to Commensal Bacteria (Loss of Tolerance)**

Human IBD patients and rodents with chronic intestinal inflammation have exaggerated humoral and cellular immune responses to commensal bacteria and/or microbial pathogens. A Th1 cytokine profile is found in CD patients (23) and most animal models. (24) On the other hand, UC evokes a mixed Th1:Th2 profile (25) and several rodent models, such as the TCRα⁻ mice, have a Th2 dominant response (26). Mechanisms of immune cell regulation largely depend on the interplay between antigen presenting cells (APC) and T lymphocytes. In normal hosts, regulatory T cells and APC inhibit pathogenic immune
responses to commensal bacteria (e.g., the phenomenon of immune tolerance) by several pathways including IL-10 and TGF-β secretion. Taken together, the observations predominantly made in animal models (see below) demonstrate (1) the importance of interacting innate and acquired immune responses, (2) the complex regulation of tolerance to luminal bacteria, and (3) the many potential mechanisms by which genetically-determined defects in immunoregulation can lead to chronic, immune-mediated intestinal inflammation.

**Animal Models of Intestinal Inflammation**

Recent advances in experimental animal models have enhanced our understanding of the contributions of mucosal immunity in IBD. The advantages of such models include the use of inbred strains of mice, as well as standardized housing conditions and diet that minimize inter-subject variation during initial onset and development of disease. The main limitation is that none of the current animal models reproduce human IBD completely. A large number of animal models exist, and they may be categorized into (a) those in which disease spontaneously arises, (b) those in which disease is induced with exogenous reagents, (c) those where disease occurs secondary to genetic manipulation, and (d) those involving transfer of immune cells to immunodeficient animals which subsequently develop disease. (27,28) (Table 3)
Table 3. Categories of animal models of intestinal inflammation based on method of induction

<table>
<thead>
<tr>
<th>Induced Spontaneous</th>
<th>Genetic</th>
<th>T-cell transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Cotton-top tamarin</td>
<td>HLA-B27/B2µ-TG rat</td>
</tr>
<tr>
<td>Immune complex/formalin</td>
<td>Cotton-top tamarin</td>
<td>IL-2&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carageenan</td>
<td>C3H/HeJ Bir</td>
<td>IL-7&lt;sup&gt;-/-&lt;/sup&gt; TG&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSS</td>
<td>Samp-1 /Yit</td>
<td>IL-10&lt;sup&gt;−/−&lt;/sup&gt;, IL-10R&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>TCRα&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>TNBS/alcohol</td>
<td>Giα2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PG-PS</td>
<td>Mdr&lt;sup&gt;1a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Oxazalone</td>
<td>N-cadherin DN</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>A20&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>TNF&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>STAT-3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>STAT-4</td>
</tr>
<tr>
<td></td>
<td>T-bet&lt;sup&gt;TG&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WASP&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>-/-</sup>, deficient, knockout; DN, dominant negative; TG, transgenic; BM, bone marrow.


Spontaneous colitis that develops in cotton top tamarins resembles IBD.(29)

Additionally, C3H/HeJ Bir mice develop predominantly right-sided colitis early in life that largely resolves by 3 months of age.(30) Experimental colitis may be induced by a variety of chemical or natural substances that damage the intestinal mucosa and produce inflammation, such as acetic acid, dextran sodium sulfate (DSS), trinitrobenzenesulfonic acid (TNBS) or oxazolone together with ethanol, peptidoglycan polysaccharide, indomethacin, or carrageenan.(31-34) Other models have been generated by gene-targeting methods to produce intestinal inflammation when rodents are housed under conventional conditions.
Several of these models utilize mice with specific mutations that affect cytokine secretion or CD4+ T cell populations. In IL-2 deficient (IL-2-/-) mice, an IL-12-driven Th1 response, characterized by high IFN-γ, results in the development of colonic inflammation with crypt hyperplasia, focal ulceration, and infiltration of the lamina propria with mononuclear cells.(35,36) Subsequent studies have suggested that CD4+ T cells, but not B cells, are critical to the pathogenesis of mucosal injury in IL-2-/- mice.(37) Similarly, IL-10 deficient (IL-10-/-) mice develop a chronic enterocolitis mediated by Th1 lymphocytes which synthesize IL-12 and pro-inflammatory cytokines such as IFN-γ and TNF.(38,39)

Colitis with crypt distortion and lamina proprial cellular infiltrate has also been observed in mice with targeted mutations of either T-cell receptor α or T-cell receptor β genes.(40) Furthermore, rats transgenic for HLA-B27 spontaneously develop a predominant CD4+ T cell-mediated colitis and gastritis when raised in conventional or SPF environments.(41) Other investigations have shown that defects in macrophage function may contribute to intestinal inflammation. In this regard, mice lacking transcription factor STAT-3 have macrophages that fail to be inhibited by IL-10, a key anti-inflammatory cytokine.(42) The requirement for a protective epithelial barrier to luminal antigens or bacteria has been demonstrated by several novel models. Mice with altered N-cadherin expression in the intestine develop patchy IBD resembling CD.(43) Other mice, having a defect in the multi-drug resistance gene (mdr-1), show intestinal inflammation similar to UC.(44) Another example includes mice lacking intestinal trefoil factor which die of extensive colitis after administration of dextran sulfate sodium.(45)

Lastly, the adoptive transfer of CD4+ T cells expressing CD45RBhigh into an immunodeficient animal (i.e., SCID mouse) causes systemic wasting and a Th1-mediated
colitis.(46) In addition, transfer of the reciprocal population, CD45RB\textsuperscript{low}, with the pathogenic CD45RB\textsuperscript{high} subset prevents colitis. Protection of colitis in this model by this CD45RB\textsuperscript{low} subset of CD4\textsuperscript{+} T cells is due to their ability to downregulate immune responses; and thus, they are referred to as regulatory T cells. Further work has shown that these regulatory cells express CD25, and exert their effects through the production of cytokines TGF-\(\beta\) and IL-10.(47-49) These studies indicate that the balance between mucosal inflammation and oral tolerance depends upon the presence of different CD4\textsuperscript{+} T cell subsets, and that the protective effects are mediated by the synthesis and release of regulatory cytokines. The importance of regulatory cytokines has been further demonstrated recently; whereas, the severity of murine colitis can be reduced when mice are given \textit{Lactococcus lactis} genetically engineered to secrete IL-10.(50)

**Role of the microflora in IBD**

Both clinical observations and animal models support the hypothesis that aberrant immune responses to resident (commensal) luminal bacteria drive chronic inflammation in genetically susceptible hosts (Table 4). Clinical improvement and decreased intestinal inflammation is observed in humans with IBD when intestinal bacteria are decreased by antibiotic administration.(51,52) Rodent models of IBD demonstrate marked reductions in clinical and histologic signs of intestinal inflammation when the animals are housed in a germ-free environment.(27,53-55) Collectively, these data indicate that inappropriate responses triggered by bacterial antigens may dictate the onset and/or maintenance of colitis as much as, or even more than, predisposing genetic factors.
Table 4. The influence of the resident bacterial environment on inflammation in animal models

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>SPF</th>
<th>Germ-Free</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Induced disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Rat</td>
<td>Acute SB, colonic</td>
<td>Attenuated acute</td>
<td>↓ by metronidazole, tetracycline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and gastric ulcers,</td>
<td>absent chronic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chronic SB ulcers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Guinea pig</td>
<td>Cecal inflammation</td>
<td>No colitis</td>
<td>↓ by metronidazole, clindamycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>Mouse</td>
<td>Colitis</td>
<td>↑ or ↓ colitis</td>
<td>↓ by cipro, imipenem</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>vancomycin, clindamycin, metronidazole</td>
</tr>
<tr>
<td>TNBS</td>
<td>Rat</td>
<td>Colitis</td>
<td>ND</td>
<td>↑ by amoxicillin, clavulanic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Genetically engineered</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-B27</td>
<td>Rat</td>
<td>Gastritis, colitis,</td>
<td>No GI or joint</td>
<td>↓ by metronidazole</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arthritis, inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>vancomycin, imipenem</td>
</tr>
<tr>
<td>C57/BL6</td>
<td>SCID</td>
<td>Colitis</td>
<td>No colitis</td>
<td>↓ by streptomycin, bacitracin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Mouse</td>
<td>Colitis, gastritis,</td>
<td>Absent attenuated inflammation</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hepatitis</td>
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<td>IL-10</td>
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</tr>
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<tr>
<td>TRα</td>
<td>Mouse</td>
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<td>No inflammation</td>
<td>ND</td>
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<tr>
<td>mdr-12</td>
<td>Mouse</td>
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<td>ND</td>
<td>↓ by streptomycin, neomycin, bacitracin, amphoteracin</td>
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<td>C. Spontaneous mutations</td>
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<tr>
<td>Samp L/Yt</td>
<td>Mouse</td>
<td>Ileitis</td>
<td>No ileitis</td>
<td>↓ by metronidazole, clindamycin</td>
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<td>Colitis</td>
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SB, small bowel; Gl, gastrointestinal; ND, not done; ↑, attenuation of disease; ↓, potentiation of disease.

Note that select models are included and that this list is not exhaustive. DSS = dextran sodium sulfate; TNBS = trinitrobenzene sulfonic acid; PG-PS = peptidoglycan-polysaccharide; WASP = Wiskott-Aldrich syndrome protein; SCID = severe combined immunodeficiency; mdr = multidrug resistance. Modified from Sartor RB. Microbial influences in inflammatory bowel diseases: role in pathogenesis and clinical implications. In: Sartor RB, Sandborn WJ, eds. *Kirsner’s Inflammatory Bowel Diseases*, 6th edn. London: Elsevier 2004, page 145.
The lumen of the distal ileum and colon contains a vast ecosystem of bacterial species, dominated by strict anaerobes which outnumber aerobic bacteria by a factor of 1000 to 1. Recent investigations suggest that resident enteric bacteria may have differing abilities to induce and perpetuate inflammation in humans and animals. Increased luminal concentrations of *Bacteroides vulgatus* and functionally altered *E. coli* have been incriminated in the pathogenesis of human IBD. *Bacteroides vulgatus* plays an essential role in the pathogenesis of carrageenan-induced colitis in guinea pigs, while sera from spontaneously colitic C3H/HeJ Bir mice show strong antibody reactivity to bacterial antigens from members of the Enterobacteriaceae and *Enterococcus* spp. Furthermore, several species of *Helicobacter* have been described, including *H. hepaticus* and *H. bilis*, which have been shown to colonize the liver and intestine and elicit IBD in immunodeficient mice and rats. These previous studies indicate a theme of host-microbial interactions and genetic regulation of host responses to commensal and pathogenic microbial agents. The diversity of animal models indicates that multiple genetic defects in immune function and disturbances in barrier integrity can lead to pathogenic host responses. Furthermore, these rodent model results suggest that each host genetic background may have a dominant bacterial stimulus and thus has important implications with regards to designing optimal therapy for individual patients. Therefore, it is important to identify which components of the indigenous microflora are responsible for the antigen-specific immune responses which contribute to the development of colitis.
The *Brachyspira hyodysenteriae* model of intestinal inflammation

*Brachyspira hyodysenteriae* is the etiologic agent of swine dysentery, an economically important disease of swine.\(^{67-69}\) Histologic lesions in pigs share similarity to those of UC in humans, and include mucosal epithelial erosions, mononuclear inflammatory cell infiltration of the lamina propria, and coagulative necrosis of the superficial mucosa.\(^{70,71}\) Putative virulence determinants of *B. hyodysenteriae* might include multiple β-hemolysin(s) and a lipopolysaccharide (LPS) or LPS-like moiety; however, the pathogenesis of the disease remains unknown.\(^{72,73}\) The disease has been reproduced in conventional pigs with pure cultures of *B. hyodysenteriae* \(^{74-76}\) and in gnotobiotic pigs co-infected with certain obligate anaerobic bacteria, including *Bacteroides fragilis*.\(^{77,78}\) Subsequent studies have demonstrated that *B. hyodysenteriae* induces gross and microscopic lesions similar to those of swine dysentery in the large intestine of mice following oral inoculation.\(^{79,80}\) Furthermore, the presence of synergistic bacteria appears to be a prerequisite for *B. hyodysenteriae* infection to express its pathogenicity in germfree pigs \(^{77,81}\) and mice \(^{77,80,82}\), suggesting that disease caused by *B. hyodysenteriae* infection may be influenced by the nature of the intestinal flora.

Gnotobiotic Studies

The luminal microenvironment may be manipulated by studying germ-free (sterile) rodents or ex-germ-free mice and rats colonized with a single (monoassociated) or a combination of bacterial species. In these studies, the severity and location of intestinal inflammation correlates closely with the magnitude of microbial stimulation.\(^{83}\) For example, IL-10\(^{−/−}\) (knockout) mice raised under conventional conditions develop lethal small
and large intestinal inflammation. However, specific pathogen free (SPF) mice have non-lethal inflammation restricted to the colon; germ-free IL-10−/− mice exhibit no clinical, histologic, or immunologic evidence of colitis. A similar lack of colitis and gastritis is found in germ-free HLA-B27/β2-microglobulin transgenic rats. Additionally, gnotobiotic B27 transgenic rats colonized with SPF bacteria develop progressively more aggressive colitis which becomes statistically significant 4 weeks after colonization. A consistent lack of colitis in other germ-free knockout and transgenic murine colitis models studied to date and in most induced models indicates the near universal requirement for commensal bacteria to induce chronic immune-mediated enterocolitis (Table 4). The acute DSS model is the only experimental model to date where the sterile environment does not markedly diminish experimental colitis although conflicting results have been reported.

Gnotobiotic studies do show that resident bacterial species have differential capacities to induce inflammation and that the dominant bacterial stimuli may depend upon the host immunologic (and perhaps genetic) background. Well recognized pro-inflammatory bacterial species include Bacteroides vulgatus, Clostridium spp., Enterococcus faecalis, certain strains of E. coli, and Helicobacter species. Importantly, the phenotype of disease in the same host is different when these different bacterial species are present. Thus, various hosts require different bacterial subsets to induce chronic immune-mediated intestinal inflammation and illustrate the complexity of interpreting clinical investigations in humans.

Lastly, host genetic susceptibility and the ability to effectively regulate host responses to resident flora contribute to IBD in humans. Polymorphisms in the leucine-rich-repeat (LRR) domain of intracellular NOD2/CARD15 are associated with CD. This domain (which is expressed in normal monocytes, macrophages, and dendritic cells, and in intestinal
epithelial cells of IBD patients) binds to LPS and peptidoglycan (especially in Gram negative bacteria) to activate the nuclear transcription factor NFκB. The NFκB signaling pathway is a key determinant of the inflammatory response (via production of pro-inflammatory cytokines such as IL-1β, TNF, IL-6, and IL-8) and maintenance of mucosal homeostasis.

**Conclusions from animal models**

Animal models have revealed that a variety of alterations in the immune response can result in predominant Th1- (53,91,92) or Th2- (33,93) induced intestinal inflammation. The recurring themes derived from these models include: (1) chronic inflammation is T lymphocyte mediated; (2) defective immunoregulatory or mucosal barrier function can induce chronic intestinal inflammation; (3) commensal enteric bacteria provide the constant antigenic and adjuvant stimuli driving chronic enterocolitis; (4) host genetic background modulates disease activity. Considerable evidence also indicates that abnormal responses to antigens in the normal bacterial flora exist suggesting that a break in oral tolerance to these microorganisms may be the trigger for chronic intestinal inflammation. This may occur due to faulty mechanisms that normally drive and regulate mucosal immunity or because of some dysfunction in the epithelial cell barrier that leads to inappropriate penetration of microbial antigens. However, the identity and mechanism(s) by which specific bacteria (or their products) interact with the host’s immune system during development of intestinal disease has not been well characterized. Gnotobiotic animals, containing a defined intestinal microflora, serve as a useful tool to search for possible microbial provocateurs of chronic immune-mediated intestinal inflammation.

**USE OF A NOVEL GNOTOBIOTIC MURINE MODEL OF COLITIS**

The long term goal of this laboratory is to investigate how the composition of the
resident enteric microflora contributes to the development of immunologically-mediated intestinal disease. The **hypothesis of this research** is that alterations in the composition of the resident enteric microflora will induce host immune responses which modulate the development of colitis. In this regard, we propose the use of a novel gnotobiotic mouse model in which host immune responses to antigens derived from the resident bacterial flora might be measured.

**Development of the *B. hyodysenteriae*-Challenge Model of Murine Typhlocolitis:**

Our research group has a longstanding interest in the investigation of microbial-host interactions; in particular, the utilization of the *Brachyspira hyodysenteriae*-challenge model to study experimentally-induced typhlocolitis in mice. Over the past 13 years, this laboratory has published on the immunopathogenesis (73,94,95), comparative effects of spirochete infection (96), mouse strain susceptibility (97), histopathological features (98), and disease development (99,100) in mice infected with *B. hyodysenteriae*. Several of these earlier investigations deserve additional commentary as they provide key observations concerning this unique model of intestinal inflammation. **Note to committee:** the genus name for the causative agent for swine dysentery has gone through several changes (i.e., *Treponema*—> *Serpulina* —> *Brachyspira*). The organism is presently placed in the genus *Brachyspira*.


The biologic activities of lipopolysaccharide-like (LPS-like, phenol/water extract) and endotoxin-like (butanol/water extract) preparations from *Brachyspira hyodysenteriae*
were examined on murine peritoneal exudate cells (PECs). Both extract preparations were less toxic than *E. coli* LPS for murine PECs. The brachyspiral butanol/water extract did induce IL-1 and TNF production but at doses 5- and 50-fold higher than that derived from *E. coli* LPS. These data indicate that butanol/water extracts from *B. hyodysenteriae* are more biologically active than the phenol/water extract and that brachyspiral endotoxin may contribute to intestinal inflammation by inducing IL-1 and TNF production.


Several inbred strains of mice were orally inoculated with *Brachyspira hyodysenteriae* B204 to determine susceptibility to infection. The influence of host genes *Ips* and *ity* on disease development were also assessed. Different inbred strains exhibited different susceptibilities to infection, with the C3H/HeN strain being the most susceptible. The *ity* gene had no apparent effect on susceptibility of infection. These data indicate that differences in disease susceptibility between mouse strains exist independent of the *Ips* locus suggesting a possible causal role for the intestinal microflora, or other environmental factors, in disease pathogenesis.


A defined diet was used to increase the susceptibility of mice infected with *Brachyspira hyodysenteriae*. Mice fed the defined diet for 7 to 14 days prior to and during the challenge period manifested more severe cecal lesions than did mice maintained on
normal rodent chow. The reason for the increase in susceptibility to \textit{B. hyodysenteriae} infection was unclear but the defined diet appeared to enhance cecal colonization by \textit{B. hyodysenteriae}. Subsequent studies (unpublished data) have shown that the diet induces a 100-1000 fold increase in total cultivable bacteria from the cecum suggesting a contributory role for gut flora in lesion development following \textit{B. hyodysenteriae} challenge.


Morphologic changes in the ceca of mice infected with either \textit{B. hyodysenteriae} or exposed to $\beta$-hemolysin were compared. Macroscopic lesions were first apparent at 14 hours post-infection while ultrastructurally, luminal bacteria were translocated through epithelial cells into the lamina propria. In other experiments, the placement of purified \textit{B. hyodysenteriae} hemolysin into surgically closed murine ceca resulted in similar ultrastructural changes as seen with \textit{B. hyodysenteriae} inoculation. Thus, the $\beta$-hemolysin of \textit{B. hyodysenteriae} causes the same early morphologic changes in the cecal mucosa of mice as are present in mice infected with the organism itself. Based on the observed bacterial translocation, luminal bacteria also appear to play a unique role in lesion development in this model.

We have since extended these earlier observations to show that the use of antibiotic therapy to selectively deplete members of the microflora (e.g., gram negative anaerobes) abolishes the ability of \textit{B. hyodysenteriae} to induce colitis.(101) \textbf{It is apparent from these investigations that the singular presence of \textit{B. hyodysenteriae} in murine ceca is}
insufficient to cause disease, and that enteric bacteria are necessary for the
development of intestinal inflammation. Apparently, the indigenous flora provides the
necessary antigenic or phlogistic stimulation to induce colitis in affected mice. Preliminary
data indicate that *B. hyodysenteriae*-infected mice develop a polarized Th1 immune response
categorized by prominent IgG2a production and IFN-γ secretion.(102) In addition,
evaluation of inflamed ceca has demonstrated that a spiral shaped bacterium significantly
invades the mucosa (18-24 hours post-infection) following infection with *B. hyodysenteriae*.
This translocated bacterium has similar morphologic features to *Helicobacter* spp. on the
basis of electron micrographs (103) (Figure 2). Analysis by polymerase chain reaction
(PCR) techniques has confirmed the presence of *Helicobacter* spp. in cecal specimens
obtained from similarly infected mice. These accumulated preliminary data strongly
incriminate normal flora (including *Helicobacter* spp.) in the pathogenesis of intestinal
inflammation in conventional mice infected with *B. hyodysenteriae*.

![Figure 2](image)

**Figure 2.** (A) Electron photomicrograph of helicobacter-like organisms in the lumen of the
mouse cecum. (B) Photomicrograph of the murine cecum obtained 24 hours following
challenge with *B. hyodysenteriae* depicting multiple helicobacter-like organisms (arrows)
within mucosal epithelial cells. Images from Hutto DL, Wannemuehler MJ. A comparison of
the morphologic effects of *Serpulina hyodysenteriae* or its beta-hemolysin on the murine
In subsequent studies, we have enhanced this model to characterize antigen-specific immune responses to a defined gastrointestinal flora in the pathogenesis of experimental colitis. Our current model utilizes gnotobiotic C3H mice harboring an altered schaedler flora (consisting of eight anaerobic organisms) which are orally infected with *B. hyodysenteriae*. Preliminary results indicate that the onset of disease is slower and lesion severity is less in gnotobiotic mice in comparison to C3H mice with a complex, conventional flora. Additionally, the introduction of a non-resident bacterium, such as *Helicobacter bilis*, into this model alters the immunological responses and lesion severity of these gnotobiotic mice subsequent to the onset of colitis. These data indicate that the nature of the gastrointestinal flora significantly influences the immunological bias of the host immune response modulating the severity of colitis.
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CHAPTER 2. COMPOSITION OF THE RESIDENT ENTERIC FLORA MODULATES THE DEVELOPMENT OF COLITIS

A paper to be submitted for publication to the journal Gastroenterology

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ABSTRACT

Background: Previous investigations from this laboratory have shown that infection of C3H strain mice with *Brachyspira hyodysenteriae* induces a typhlocolitis with features that are similar to ulcerative colitis. The immunological response of these conventional C3H/HeOuJ mice has been shown to be a predominant Th1-like response (e.g., antigen-specific IgG2a responses and IFN-γ responses). While the spirochetal pathogen initiates the onset of disease, this model of bacterial-induced colitis has been shown to be dependent upon the nature of the resident bacterial flora and the host’s inflammatory response. **Aim:** To characterize antigen-specific immune responses to a defined gastrointestinal flora in the pathogenesis of experimental colitis. **Methods:** Gnotobiotic C3H/HeN mice possessing an altered schaedler flora (8 separate organisms) were used to evaluate the host’s response following challenge with *B. hyodysenteriae*. Immunologic parameters evaluated included altered cecal morphology, histologic lesion severity scores, isotype of the antigen-specific serum antibody (ELISA), and antigen-induced cytokine responses. **Results:** Results indicate that the onset of disease is slower and lesion severity is less in the gnotobiotic mice in comparison to C3H mice with a complex, conventional flora. The immunologic response of the gnotobiotic mice subsequent to onset of disease was of mixed Th1:Th2 phenotype, but predominantly Th2-like. Because the conventional colony of C3H mice was colonized with *Helicobacter* species, we co-infected the gnotobiotic C3H mice with *H. bilis* to assess the influence of helicobacters on the severity of disease and the bias of the immune response. Results indicate that colonization of the gnotobiotic C3H mice with *H. bilis* did enhance the severity of the colonic lesions but that the immune response was shown to be Th2-like. **Conclusions:** These data indicate that the nature of the gastrointestinal flora significantly
influences the immunologic bias of the immune response and the presence of *Helicobacter* species do not apriori predispose toward a Th1 immune response. The *B. hyodysenteriae* challenge model provides reproducible typhlocolitis quickly and consistently in immunocompetent mice and can be used to assess the host’s immune response following onset of colitis.
Introduction

Aberrant or exaggerated immune responses to bacterial antigens derived from the intestinal lumen are thought to serve as the initiating events leading to the development of inflammatory bowel disease (IBD).\textsuperscript{1} For example, humans who express the HLA-B27 haplotype are known to be predisposed to colitis.\textsuperscript{2} The recent observation that germfree, HLA-B27 transgenic rats fail to develop colitis suggests that host immunologic responses to bacterial antigens derived from the intestinal microflora are a part of the pathogenic mechanism.\textsuperscript{3,4} The importance of immune responsiveness to normal bacterial antigens in the pathogenesis of disease has additionally been demonstrated using genetically manipulated (i.e., knock-out) mice (e.g., IL-2-, IL-10-, T-cell receptor-\(\alpha\) deficient mice).\textsuperscript{1} Collectively, these and other observations support the hypothesis that unregulated or inappropriate responses to normal resident microflora may dictate the initiation or perpetuation of chronic intestinal inflammation as much as, or even more than, predisposing genetic factors. This contention is supported by observations that genetic disruptions in the immune response are not pathognomonic in themselves as is demonstrated when these mice are reared under gnotobiotic or specific-pathogen free (SPF) conditions.\textsuperscript{3,5,6} In addition, humans with IBD and experimental models with IBD enter a period of remission following antibiotic therapy.\textsuperscript{1,7-11} However, the unequivocal endorsement of this concept has been clouded by the use of experimental models relying on spontaneous development, as well as, difficulties in ascertaining whether the observed colitis has been directly mediated by acute inflammatory responses or acquired immune responses. In order to distinguish between these possibilities, a predictable and highly reproducible model by which microbial-induced inflammation can be initiated and examined is needed.
Previously, we have shown that infection of C3H mice with *Brachyspira hyodysenteriae* induces a typhlocolitis with features that are similar to ulcerative colitis.\textsuperscript{12,13} The immunological response of these conventional C3H/HeOuJ mice was shown to be predominantly Th1-like (e.g., antigen-specific IgG2a and IFN-\(\gamma\) responses). While the spirochete, *B. hyodysenteriae*, initiates the onset of disease, this model of bacterial-induced colitis is dependent upon the nature of the resident bacterial flora and the host’s inflammatory response.\textsuperscript{14} In addition, evaluation of inflamed ceca has demonstrated that a spiral shaped bacterium significantly invades the mucosa (18-24 hours post-infection) following infection with *B. hyodysenteriae*.\textsuperscript{12} In subsequent studies, we have utilized electron microscopy and polymerase chain reaction (PCR) techniques to confirm that the translocated bacteria are *Helicobacters*.\textsuperscript{15} These accumulated preliminary data strongly incriminate the normal microflora (including *Helicobacter* spp.) in the pathogenesis of intestinal inflammation in conventional mice infected with *B. hyodysenteriae*.

To better understand the nature of microbial populations inciting intestinal inflammation, we first infected gnotobiotic C3H mice (harboring an altered schaedler flora composed of eight separate organisms) with *H. bilis* followed by co-infection 3 weeks later with *B. hyodysenteriae*. We addressed the hypothesis that alterations of the resident microflora modulate the susceptibility to the development of experimental colitis. Results indicate that the onset of disease is slower and lesion severity is significantly less in gnotobiotic mice in comparison to C3H mice with a complex, conventional flora. Moreover, the introduction of a non-resident bacterium, such as *H. bilis*, into this model system alters the immunological responses and lesion severity of these gnotobiotic mice subsequent to the onset of colitis. These observations indicate that the composition of the enteric microflora
influences the immunological bias of the host response to luminal bacteria and mediates the intensity of colitis.

**Materials and Methods**

**Animals.** Four- to eight-week old gnotobiotic reared C3H/HeN mice (mostly female) were obtained from Taconic Farms (Germantown, N.Y.). The gnotobiotic mice were colonized with an altered schaedler flora (ASF) consisting of eight anaerobic bacteria (Table 1). Gnotobiotic mice were housed in Trexler plastic isolators under sterile conditions and fed an irradiated diet (e.g., Harlan-Teklad) and autoclaved water for the duration of the project. All mice were certified free of *Helicobacter* spp. by the vendor and re-tested on-site prior to study enrollment. All manipulations performed on mice were approved by the Iowa State University Committee on Animal Care.

<table>
<thead>
<tr>
<th><strong>Taxon</strong></th>
<th><strong>Strain</strong></th>
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<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>ASF 360</td>
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<tr>
<td><em>Lactobacillus animalis</em></td>
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</tr>
<tr>
<td><em>Bacteroides distasonis</em></td>
<td>ASF 519</td>
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<td><em>Flexistipes</em> phylum</td>
<td>ASF 457</td>
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<td>ASF 356</td>
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<tr>
<td><em>Eubacterium plexicaudatum</em></td>
<td>ASF 492</td>
</tr>
<tr>
<td>Low G + C-content spp.</td>
<td>ASF 500</td>
</tr>
<tr>
<td><em>Clostridium</em> cluster XIV</td>
<td>ASF 502</td>
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**Table 1.** Bacterial members comprising the altered schaedler flora (ASF).

**Treatment Groups and Study Design.** Gnotobiotic mice were assigned to one of four treatment groups on the basis of *H. bilis* infection and *B. hyodysenteriae* inoculation as follows: Group 1 (control) - no infection; Group 2 - infected with *B. hyodysenteriae* alone; Group 3 - infected with *H. bilis* alone; Group 4 - dual-infected with *H. bilis* and with *B.*
*hyodysenteriae*. Representative experimental and control mice were euthanized by CO₂ asphyxiation on *B. hyodysenteriae* post-infection day 21, with samples processed for gross lesions, bacteriology, histopathology, antigen-specific serum IgG isotype concentrations, and levels of cytokines secreted from antigen stimulated cells.

**Bacterial Inoculation.** Mice were orally infected with a bacterial inoculum (0.3 ml, approximately $10^8-10^9$ CFU/ml) of *H. bilis* (ATCC strain 51630) administered every other day for three doses. Dual-infected mice received the *H. bilis* inoculum 3 weeks prior to challenge with *B. hyodysenteriae*. Confirmation of *H. bilis* infection post-inoculum was made by PCR evaluation of feces or cecal contents and culture. Dual-infected mice were inoculated with $10^8$ CFU/ml *B. hyodysenteriae* organisms given by orogastric intubation. Previous experiments utilizing conventional C3H mice had indicated that characteristic histological lesions were evident 72 to 96 hours post-inoculation, with the spirochete infection confirmed at necropsy by demonstration of organisms grown on selective media.

**Helicobacter bilis and ASF-Specific PCR.** Fecal samples or cecal contents were analyzed for *Helicobacter* spp and *H. bilis* as described previously. For generic *Helicobacter* PCR, 5μl of quantitated fecal DNA was used as template while for *H. bilis* PCR, 10μl of quantitated fecal DNA was used as template for all samples. Terminal confirmation of ASF colonization was performed by PCR of fecal pellet DNA from defined flora mice using ASF-specific primers, with modifications made to some primer sequences and the PCR conditions.

**Bacterial Antigens.** The *H. bilis* organisms were cultivated in Columbia broth and the bacteria were harvested by centrifugation. The cells were washed with sterile PBS, the cell
pellet was frozen, cells were lyophilized, and then stored at \(-20^\circ\text{C}\) until used. *Brachyspira hyodysenteriae* B204 was grown anaerobically in trypticase soy broth supplemented with 2 % horse serum. Cells were grown overnight and harvested by centrifugation. Subsequently, cells were washed with buffer, lyophilized, and stored at \(-20^\circ\text{C}\) until further use. Each member of the altered schaedler flora (ASF 356, ASF 360, ASF 361, ASF 457, ASF 492, ASF 500, ASF 502, and ASF 519) was grown in anaerobically prepared medium and cultured at \(37^\circ\text{C}\) for five to seven days. Cells were grown in either reinforced clostridium medium (RCM) supplemented with 5 % calf serum (ASF 360, ASF 361, ASF 356, ASF 502, and ASF 519) or schaedler broth supplemented with 5 % sheep serum (ASF 457, ASF 492, and ASF 500). Because many of these organisms are extremely oxygen sensitive (EOS), all inoculations were performed in a Coy anaerobic chamber. Culture flasks were sealed in anaerobic jars and then placed in an incubator. As above, the cells were harvested from broth by centrifugation, washed in PBS, and lyophilized.

Bacterial antigens (e.g., *H. bilis*, *B. hyodysenteriae*, and all eight ASF strains) used for ELISA or for stimulation of lymphocyte cultures were prepared from the lyophilized cells. Briefly, cells were weighed and resuspended in buffer or tissue culture medium to a final concentration of 2 mg/mL (dry weight to volume). The cell suspension was then placed on ice and sonicated for 5 minutes to prepare whole cell sonicates (WCS). Sonication was performed using 30 second pulses to prevent the suspension from overheating (i.e., denaturing of the proteins). Cell disruption was monitored microscopically. Protein content was determined by BCA analysis per manufacturer's instructions (Pierce Laboratories). For the preparation of antigen for in vitro stimulation of lymphocytes, the WCS were sterilized by UV light. Additionally, sterility was confirmed by bacteriological methods (e.g., aerobic
and anaerobic blood agar cultures). These methods were used to prepare all antigens used for these studies. In order to ensure that all studies were performed with the same lot of antigens, each of the bacterial species to be used was grown during the first six months of the project to provide sufficient antigenic mass to complete these experiments.

Gross Pathology. Macroscopic cecal lesions were scored using previous published criteria. Briefly, ceca were evaluated macroscopically for atrophy, edema, and/or increased mucus in the ingesta, and given a gross lesion score (range 0-3: 0 = normal, 3 = severe cecal lesion). Representative tissue sections were fixed in neutral buffered 10% formalin for histopathologic evaluation.

Histology. Formalin-fixed cecal tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for assessment of inflammation. Tissue sections were coded to blind the pathologist (J Hostetter, Dept. of Veterinary Pathology) to the infection status of the animal. The cecal tissue sections from each mouse were scored on severity of mucosal epithelial damage, architectural/glandular alterations, and magnitude/character of lamina propria cellular infiltrate (Table 2).
Table 2. Scoring System for Histopathological Evaluation of Gastrointestinal Inflammation

<table>
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<tr>
<th>Parameters</th>
<th>Scoring Criteria</th>
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<tr>
<td>Mucosal height</td>
<td>Ratio of crypt depth to width. Rising score from 1 to 5 (mild to severe hyperplasia)</td>
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<tr>
<td>LP immune cells</td>
<td>Lymphocytes/plasma cells are the expected population. Mixed inflammation = increased neutrophils</td>
</tr>
<tr>
<td>Erosions</td>
<td>Rising score from 1-5 (0 = no erosions; 5 = ulceration)</td>
</tr>
<tr>
<td>Inflammatory score</td>
<td>Denotes quantity of cellular infiltrates in the LP. Rising score from 1-5 (normal to severe inflammation with necrosis)</td>
</tr>
<tr>
<td>Edema score</td>
<td>Rising score from 1-5 (1 = mild edema; 5 = severe edema with architectural distortion)</td>
</tr>
<tr>
<td>Other</td>
<td>Submucosal lymphoid hyperplasia (present or absent)</td>
</tr>
</tbody>
</table>

Table 2. Scoring system for the histopathological evaluation of gastrointestinal inflammation. LP = lamina propria.

Antigen-Specific Serum Antibodies. Blood samples were obtained via cardiac puncture at the same time tissues were collected for analysis. Resulting sera samples were stored at -20°C until assays were performed. Determination of *H. bilis*, *B. hyodysenteriae*, and ASF-specific antibodies in sera was performed by ELISA as previously described. Briefly, 96-well microtiter plates (Immunlon II) were coated with sonicated whole cell lysates of each of the eight anaerobic bacteria comprising the altered schaedler flora, *H. bilis*, and *B. hyodysenteriae*. Diluted serum (1:200) was added to each well and incubated at 4°C overnight. Then, alkaline phosphatase conjugated goat anti-mouse IgG1 or IgG2a was added and incubated for 1 hour at room temperature. Wells were colorimetrically developed using p-nitrophenyl phosphate at room temperature. Optical densities were measured at 405 nm using a microplate reader.

Cytokine Analysis by Multiplexed Flow Cytometric Assay. Lymphocytes isolated from
the spleen and mesenteric lymph nodes were incubated at $2.5 \times 10^6$ cells/ml with or without whole cell lysates of the ASF organisms, *H. bilis*, and *B. hyodysenteriae* for 72 hours. Cell cultures were treated with concanavalin A to evaluate the overall nature of the T cell response. Cell-free supernatants were harvested and analyzed for their concentration of Th1 (TNF-α, IFN-γ, IL-2, IL-12) and Th2 (IL-4, IL-10) cytokines using a multiplexed flow cytometric assay (The FlowMetric™ System, Luminex, Austin, TX). Cytokine concentrations were measured using a murine cytokine detection kit (Mouse Cytokine LINCOplex Kit, Linco Research, St. Charles, MO) specifically designed for use with the Luminex systems.

**Data Analysis.** All data are expressed as mean ± SEM with values derived from 3 independent experiments. For all analyses, non-parametric assays were utilized with a $P<0.05$ considered significant.

**Results**

**Colonization with *H. bilis*, *B. hyodysenteriae*, and ASF bacteria**

Mice inoculated with *H. bilis* became positive by fecal PCR 12-14 days post-infection. Both *Helicobacter* genus and *H. bilis*-specific primers consistently detected infection under optimal conditions. In some instances, culture of cecal contents at necropsy was also used to confirm colonization with *Helicobacter* spp. Fecal samples recovered from uninfected control animals remained PCR negative for *H. bilis*. Confirmation of infection with *B. hyodysenteriae* was made from feces collected from infected mice, with the organism subsequently grown on selective media. Specific PCR products for members of the ASF (with the exception of that derived from ASF member 360) were reliably obtained by
amplification of feces or cecal contents obtained at necropsy from defined flora mice using the ASF-specific primers in a multiplex format (data not shown).\textsuperscript{16}

Gnotobiotic mice develop typhlocolitis of varying severity subsequent to colonization by *H. bilis* and *B. hyodysenteriae*

Clinical signs of colitis (e.g., diarrhea) were variably observed in dual-infected defined flora (DF) mice and those colonized with *B. hyodysenteriae* alone. Cecal scores from these groups were significantly (*P*<0.05) increased as compared to scores in defined flora and *H. bilis*-infected groups (Table 3). Grossly, diseased ceca had thickened cecal walls and contained pasty contents. In contrast, *H. bilis*-infected mice had only mild inflammation characterized by cecal involution and local mesenteric lymphadenopathy. Control mice had no detectable lesions.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cecal score</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defined flora</td>
<td>0</td>
<td>Grossly normal</td>
</tr>
<tr>
<td><em>H. bilis</em> infected</td>
<td>0.2 ± 0.08</td>
<td>Ceca slightly smaller</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mesentric lymphadenopathy</td>
</tr>
<tr>
<td><em>B. hyodysenteriae</em> infected</td>
<td>1.4 ± 0.1\textsuperscript{a}</td>
<td>Moderate to marked edema</td>
</tr>
<tr>
<td>Dual infected</td>
<td>1.4 ± 0.1</td>
<td>Small ceca / mucoid contents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate edema</td>
</tr>
</tbody>
</table>

\textbf{Table 3.} Gross Pathologic Observations in Gnotobiotic Mice

\textsuperscript{a} *P*<0.05 versus defined flora and *H. bilis*-infected mice.

\textbf{Table 3.} Gross pathological scores of gnotobiotic mice infected with or without *H. bilis* or *B. hyodysenteriae*. Numbers reflect mean scores ± SEM derived from 6-8 mice per treatment group averaged over 3 independent experiments. Maximum score is 3.0 (See Methods for scoring criteria).\textsuperscript{a}
Similar to the gross observations, histologic lesions in dual-infected DF mice and mice infected with *B. hyodysenteriae* were significantly (*P*<0.05) increased over controls and *H. bilis*-infected littermates (Table 4). Cecal inflammation in *H. bilis*-infected mice was characterized by predominantly mononuclear infiltration of the lamina propria (LP) with scattered foci of neutrophils. A similar but more intense infiltrate (accompanied by increased mucosal edema) was observed in *B. hyodysenteriae*-infected DF mice (Figure 1, panel C). Cecal tissues from dual-infected DF mice showed slightly more inflammation than *B. hyodysenteriae*-infected mice, with even greater LP cellular infiltrate, increased mucosal height, crypt hyperplasia, and mucosal edema (Figure 1, panel D). Tissue sections of ceca from non-infected control mice were histologically normal.

### Table 4. Histopathological Observations in Gnotobiotic mice

<table>
<thead>
<tr>
<th>Intestinal colonization</th>
<th>Histologic score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defined flora</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td><em>H. bilis</em></td>
<td>5.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>9.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dual infected</td>
<td>9.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Maximum value for each parameter except that assigned to inflammatory cells is 5, and maximum total histology score is 20.

<sup>b</sup>*P*<0.05 for *H. bilis* versus defined flora and *B. hyodysenteriae* or dual infected.

<sup>c</sup>*P*<0.05 for *B. hyodysenteriae* or dual versus *H. bilis* and defined flora.
Figure 1. Histological changes in the cecal mucosa of defined flora mice after bacterial infection. (A) Controls – no infection; (B) Mice infected with *H. bilis* alone; (C) Mice infected with *B. hyodysenteriae* alone. Note the severe mucosal edema, cellular infiltrate, and diffuse epithelial erosions; (D) Mice infected with both *H. bilis* and *B. hyodysenteriae*. All images (160X) at PI-day 42.
Gnotobiotic mice develop antigen-specific IgG1/IgG2a responses to the resident microflora following colonization with \textit{H. bilis} and \textit{B. hyodysenteriae}

Elevated serum concentrations of IgG1 and IgG2a antibodies directed against specific members of the ASF were observed in DF mice colonized with either novel bacterium, and these responses were greatest in \textit{H. bilis}-infected mice (Figure 2A). The evaluation of pooled antibody responses revealed significant group differences in IgG isotype levels directed at antigens derived from members of the resident flora (Figure 2A). For IgG1, all colonization groups evoked significantly ($P<0.05$) increased anti-\textit{H. bilis} antibody responses compared to control mice, with the greatest response observed in \textit{H. bilis}-infected mice (Figure 2B). Similarly, both \textit{H. bilis} and dual infected mice had significantly ($P<0.05$) increased IgG2a responses directed against \textit{H. bilis} antigen when compared to \textit{B. hyodysenteriae}-infected mice and non-infected controls (Figure 2B).
Figure 2A. Antibody responses to antigens derived from members of the altered Schaedler flora (ASF). Serum was collected from gnotobiotic mice 21 days following challenge with *B. hyodysenteriae*. The serum samples from individual mice were diluted (1:200) and analyzed individually by ELISA using sonicated whole cells of ASF bacteria as antigen. Numbers reflect means ± SEM of the optical density (absorbance) for each treatment group. Each mean value comprises triplicate wells of individual mouse serum from 4-8 mice per treatment group averaged over 3 independent experiments. The control group consisted of gnotobiotic mice colonized with the 8 organisms comprising the ASF; the *H. bilis* group was infected with *H. bilis* on day -21; the *B. hyodysenteriae* group were gnotobiotic mice colonized with *B. hyodysenteriae* on day 0; and the dual-infected group were mice infected with both *H. bilis* (day -21) and *B. hyodysenteriae* (day 0). ASF 356 = Clostridium cluster XIV; ASF 360 = *Lactobacillus acidophilus*; ASF 361 = *Lactobacillus animalis*; ASF 457 = *Flexistipes* phylum; ASF 492 = *Eubacterium plexicaudatum*; ASF 500 = Low G + C-content spp.; ASF 502 = *Clostridium* cluster XIV; ASF 519 = *Bacteroides distasonis*.
Figure 2B. Pooled antibody responses to antigens of the altered schaedler flora (ASF). Serum was collected from gnotobiotic mice 21 days following challenge with \textit{B. hyodysenteriae}. The serum samples from individual mice were diluted (1:200) and analyzed individually by ELISA using sonicated whole cells of ASF bacteria as antigen. Numbers reflect means ± SEM of the pooled optical density (absorbance) for all 8 ASF antigens averaged over 3 independent experiments. The control group consisted of gnotobiotic mice colonized with the 8 organisms comprising the ASF; the \textit{H. bilis} group was infected with \textit{H. bilis} on day -21; the \textit{B. hyodysenteriae} group were gnotobiotic mice colonized with \textit{B. hyodysenteriae} on day 0; and the dual-infected group were mice infected with both \textit{H. bilis} (day -21) and \textit{B. hyodysenteriae} (day 0). ASF 356 = Clostridium cluster XIV; ASF 360 = \textit{Lactobacillus acidophilus}; ASF 361 = \textit{Lactobacillus animalis}; ASF 457 = \textit{Flexistipes} phylum; ASF 492 = \textit{Eubacterium plexicaudatum}; ASF 500 = Low G + C-content spp.; ASF 502 = \textit{Clostridium} cluster XIV; ASF 519 = \textit{Bacteroides distasonis}. Statistical inferences are noted under each histogram in Figure 2B.
Colonization of gnotobiotic mice with *H. bilis* or *B. hyodysenteriae* evokes compartmentalized cytokine responses to members of the resident microflora

Cytokine responses in lymphocyte culture supernatants from the spleen (SPL) and mesenteric lymph nodes (MLN) were compartmentalized in their magnitude and varied between treatment groups (Figure 3A). Responses in SPL supernatants were greatest for IL-4 and IL-10 while IFN-γ responses were greater in MLN cultures. When analysis of pooled cytokine responses was performed, significant (*P*<0.05) differences between groups were observed for all cytokines in both SPL and MLN cultures (Figure 3B). The details of these inferences are included under each histogram of Figure 3B. Supernatants derived from lymphocyte cultures from non-infected DF mice showed minimal but detectable levels (pg/ml) of IFN-γ, IL-4, and IL-10.
Figure 3A. Detection of cytokine production to individual ASF antigens in culture supernatant fluid from splenocytes (SPL) or mesenteric lymph node (MLN) cells. Lymphocytes recovered from mice in each of four treatment groups were cultured in the presence of specific antigens (25 μg/mL) or concanavalin A (5 μg/mL) for 72 hrs and harvested for analysis. Undiluted supernatant fluid was analyzed by multiplexed flow cytometric assay for the amount of IFN-γ, IL-10 or IL-4. Data represent means ± SEM of cytokine concentration (pg/ml) derived from 3 independent experiments. 360 = Lactobacillus acidophilus; 492 = Eubacterium plexicaudatum; 500 = Low G + C-content species; 502 = Clostridium cluster XIV; 519 = Bacteroides distasonis; H. bilis = Helicobacter bilis; B. hyo = Brachyspira hyodysenteriae B204.
Figure 3B. Detection of pooled cytokine responses to ASF antigens per group in culture supernatant fluid from splenocytes (SPL) or mesenteric lymph node (MLN) cells following antigenic stimulation. Lymphocytes recovered from mice in each of four treatment groups were cultured in the presence of specific ASF antigens (25 µg/mL) for 72 hours and harvested for analysis. Undiluted supernatant fluid was analyzed by multiplexed flow cytometric assay for the amount of IFN-γ, IL-10 or IL-4. Data represent means ± SEM of the pooled cytokine concentrations (pg/ml) to all 8 ASF antigens per group derived from 3 independent experiments. Statistical inferences are noted under each histogram in Figure 3B.
Discussion

The *B. hyodysenteriae*-challenge model of colitis is unique and differs from most other mouse models. Its distinct advantages include (a) the initiating etiologic agent (*B. hyodysenteriae*) is known; (b) a defined bacterial flora (gnotobiotic or conventional) is necessary to develop intestinal inflammation; (c) this model provides reproducible typhlocolitis quickly and consistently allowing temporal studies to be performed; and (d) immunocompetent mice are used to assess the host’s immune response following onset of colitis. We have previously reported on the strain-related differential susceptibility in conventional mice to *B. hyodysenteriae* infection and on the use of a defined diet to enhance the susceptibility of mice to the pathogenic effects of the spirochete infection.\(^{13,17}\) We have since extended these observations to show that the use of antibiotic therapy to selectively deplete members of the microflora (e.g., gram negative anaerobes) abolishes the ability of *B. hyodysenteriae* to induce colitis.\(^{13,23}\) It is apparent from these investigations that the singular presence of *B. hyodysenteriae* in murine ceca is insufficient to cause disease, and that enteric bacteria are necessary for the development of intestinal inflammation. Apparently, the indiginoous flora provides the necessary antigenic or phlogistic stimulation to induce colitis in affected mice.

Previous studies demonstrate a crucial role for resident bacteria in the pathogenesis of experimental colitis and inflammatory bowel disease (IBD) in humans.\(^{1,24,25}\) Both spontaneous and induced inflammation in a diverse range of rodent models have been associated with commensal resident bacteria.\(^{3,6,26-35}\) Determining the nature of those microbial populations that are responsible for the initiation and perpetuation of colitis is difficult given the complex microbial environment present in most models. Importantly, not
all luminal bacteria have equivalent abilities to induce inflammation. *Bacteroides vulgatus* induces colitis in carrageenan-fed guinea pigs\(^{32}\) and HLA-B27 transgenic rats\(^{3,36}\) and induces a comparable degree of colitis in monoassociated HLA-B27 transgenic rats to lesions observed in transgenic rats colonized with six bacterial species (including *B. vulgatus*).\(^{36}\) Both HLA-B27 transgenic rats monoassociated with *E. coli* and IL-10\(^{-/-}\) mice monoassociated with *B. vulgatus, Helicobacter hepaticus, Clostridium sordelii* or viridans-type streptococci fail to develop colitis.\(^{37,38}\) On the other hand, *Enterococcus faecalis* induces IBD in IL-10\(^{-/-}\) mice.\(^{39}\) Thus different bacteria and/or their products are required to evoke chronic immune-mediated inflammation in various susceptible hosts.

The gnotobiotic mouse model is a useful tool for evaluating the roles of bacterial species in the development of intestinal inflammation. Our rationale was that the use of an available defined flora mouse model that is more complex, and seemingly more realistic, than monoassociated models would assist in the study of microbially-triggered host responses that predispose to or mediate intestinal inflammation.\(^{16}\) We chose to introduce *H. bilis* into the *B. hyodysenteriae*-challenge model as a novel non-resident bacterium (e.g., provocateur), based on its role as a potential pathogen in conventional mice infected with *B. hyodysenteriae* and based on the association of *Helicobacter* spp. with IBD in other rodent models.\(^{35,40-44}\) It was anticipated that intestinal inflammation would be mediated by a pro-inflammatory Th1 immune response (based on previous studies in conventional flora mice colonized with *B. hyodysenteriae*) directed at members of the resident microflora, and that this response would be potentiated following the introduction of *H. bilis*. While we did observe modest increases in gross/histologic inflammatory indices in dual-infected mice, the ability of *H. bilis* infection to modulate immunologic responses (e.g., to a balanced Th1:Th2
profile) to resident microflora at a single time point was an unexpected finding. This observation suggests that the introduction of a previously non-resident bacterium may alter the homeostatic balance of the commensal flora and evoke antigen-specific responses capable of inciting an inflammatory response in a susceptible host.

Some earlier studies suggest that infection with some *Helicobacter* species evokes Th1-mediated chronic gastrointestinal inflammation.\textsuperscript{45-47} In the present study, the profile of antibody and cytokine responses to antigens derived from the resident flora were of mixed Th1:Th2 expression, but with Th2 responses predominating in the *Helicobacter* groups. We observed only mild gross and histologic lesions in *H. bilis*-infected mice while more severe colitis was seen in *B. hyodysenteriae*- and dual-infected mice. While these observations demonstrate that *B. hyodysenteriae* can induce cecal inflammation in gnotobiotic mice, they also suggest that additional bacterial interactions (e.g., introduction of *H. bilis* into the intestinal microflora) contribute to the genesis of host immunologic responses.\textsuperscript{3} We hypothesize that colonization with *H. bilis* perturbed the local microecology and influenced the immunological bias of the host response to luminal bacteria (i.e., broke tolerance or overcame immunological ignorance). In turn, these protective Th2 responses generated against commensal members, as a consequence of *H. bilis* colonization, mitigated lesion severity in dual-infected mice by apposing the pro-inflammatory (e.g., Th1-like) responses characteristic of *B. hyodysenteriae* infection alone.

The profile of antibodies and cytokines in gnotobiotic mice having different compositions of resident microflora suggest that subpopulations of luminal bacteria exert a dominant influence on the induction of subsequent immune responses. Elevated serum levels of IgG1 and IgG2a to diverse ASF members (in particular ASF356, ASF360, ASF457) were
observed in *H. bilis*, *B. hyodysenteriae*, and dual-infected groups, with the greater antibody responses seen in *H. bilis*-infected mice. Increased concentrations of IgG directed against luminal commensal bacteria have been reported in several different animal models of colitis. Defined flora mice colonized with *H. bilis* produced greater serum IgG1 and IgG2a antibody responses to *H. bilis* (relative to the responses to the 8 ASF) similar to that described in other *Helicobacter*-associated IBD models. Induction of antigen-specific secretion of IL-4, IL-10 and IFN-γ were differentially present in lymphocyte cultures of gnotobiotic mice. Relative to non-infected control mice, we observed increased Th1/Th2 cytokine concentrations between the different mouse groups with *B. hyodysenteriae*- and dual-infected mice evoking the greatest cytokine responses.

The compartmentalized Th2-like cytokine responses present in dual-infected mice, characterized by increased IL-4 and IL-10 in splenic cultures but decreased IFN-γ in MLN cultures are noteworthy, especially when these results are compared to those findings in *B. hyodysenteriae*-infected mice. Maggio-Price et al have recently reported decreased IFN-γ transcripts in colonic tissues of *H. hepaticus*-infected *mdr* 1a−/− mice having mild lesions of IBD. The decreased IFN-γ secretion in dual-infected mice was surprising but may be attributable to the effects of *H. bilis* infection which may down-regulate this pro-inflammatory cytokine and lessen its role in promoting colitis in a susceptible murine host. In keeping with this Th2 profile of cytokines, we found that spleen cells recovered from dual-infected mice also secreted greater concentrations of IL-10 following antigenic stimulation than cells recovered from the other three groups of mice. Cecal bacterial lysates or LPS may induce high concentrations of IL-10 by murine mesenteric lymph node cells or splenocytes with most being produced by macrophages or dendritic cells. IL-10 is an important
cytokine in modulating inflammatory responses in IBD, likely through T regulatory cells (Tr1) and also by B cells and dendritic cells.\textsuperscript{35,53-56} The anti-inflammatory actions of IL-10 include inhibition of antigen-specific proliferation and cytokine production by Th1 lymphocytes and down regulatory effects on antigen presenting cells via suppression of macrophage activation and IL-12 production.\textsuperscript{56-59} Kullberg et al\textsuperscript{45} showed that CD4\textsuperscript{+} T cells from \textit{H. hepaticus}-infected wildtype mice could prevent colitis in Rag\textsuperscript{2-} mice when co-transferred with CD4\textsuperscript{+} T cells from \textit{H. hepaticus}-colonized IL-10\textsuperscript{-/-} mice; and that this antigen-specific protection was mediated through the secretion of IL-10. However, IL-10 may have diverse roles in mediating \textit{Helicobacter}-induced inflammation since increased IL-10 levels accompany severe IBD in \textit{mdr} 1a\textsuperscript{-/-} mice with or without \textit{H. bilis} infection but levels are not increased in \textit{H. hepaticus}-infected \textit{mdr} 1a\textsuperscript{-/-} having delayed IBD development.\textsuperscript{35}

These results indicate that perturbation of the microflora may evoke antigen-specific immune responses to nonpathogenic bacteria but they do not completely explain those bacterial components responsible for host responses. Both adjuvants and antigens derived from commensal enteric bacteria may stimulate cell-mediated immune responses. Luminal bacteria produce several non-viable bacterial products (e.g., LPS\textsuperscript{1} [from Gram-negative bacteria], peptidoglycan-polysaccharide (PG-PS) complexes\textsuperscript{60} [from both Gram-positive and Gram-negative bacteria], and bacterial DNA\textsuperscript{61} [containing CpG motifs]) which can stimulate innate immunity through pattern recognition receptors, especially toll-like receptors (TLR).\textsuperscript{62-64} Bacterial flagellin, an immunodominant antigen present in several ASF bacteria (ASF502, ASF492) as well as flagellin from some \textit{Treponema} spp., may also stimulate pathogenic intestinal immune reactions through TLR 5.\textsuperscript{65} Serum and mucosal
antibody responses likely indicate increased mucosal permeability and/or enhanced immunologic responsiveness to multiple resident species. Increased levels of inflammatory cytokines, such as IFN-γ, may also impair epithelial barrier integrity and contribute to mucosal inflammation. Furthermore, Helicobacter spp. infection may initiate the development of intestinal inflammation through diverse mechanisms including the elaboration of specific toxins (e.g., cytolethal distending toxins), IFN-γ-mediated compromise of intestinal epithelial barrier function, the production of commensal-specific CD4+ T cells, LPS-mediated activation of innate immunity through TLR, and/or altered epithelial cell gene expression. It is likely that host responses to commensal luminal bacteria are quite heterogeneous and that numerous mechanisms contribute to intestinal inflammation.

In summary, our study describes the use of a gnotobiotic model to investigate the nature of microbial populations inciting intestinal inflammation. The introduction of a novel bacterium (H. bilis and/or B. hyodysenteriae) perturbs the local microbial environment and evokes an array of antigen-specific host responses against commensal bacterial flora. While it was anticipated that gnotobiotic mice would produce Th1 immune responses to commensal flora following colonization by one or both novel bacteria, instead it was shown that H. bilis-infected and dual-infected mice produced predominant serum IgG1 and IL-10 but decreased locally-derived IFN-γ, suggestive of a Th2 immune response. These findings indicate that the nature of the gastrointestinal flora significantly influences the immunologic bias of the host response and the presence of Helicobacter species does not apriori predispose toward a Th1 inflammatory response.
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CHAPTER 3. GNOTOBIOTIC MICE COLONIZED WITH *HELCOBACTER BILIS* DEMONSTRATE ANTIGEN-SPECIFIC IMMUNE RESPONSES TO RESIDENT ENTERIC FLORA

A paper to be submitted for publication to the journal *Gut*

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ABSTRACT

Background and Aims: Infection with Helicobacter species has been associated with the development of mucosal inflammation and inflammatory bowel disease (IBD) in several mouse models. However, consensus regarding the role of Helicobacter as a potential pathogen or its utility as a model organism to study intestinal inflammation is confounded by the presence of a complex colonic microbiota. The aims of this study were to determine whether colonization of gnotobiotic mice with Helicobacter bilis provokes an antigen-specific immune response to the resident enteric flora, and to investigate the kinetics of this response. Methods: Gnotobiotic C3H/HeN mice possessing a defined flora (e.g., altered schaedler flora consisting of 8 intestinal bacteria) were used to evaluate the host’s response following colonization with H. bilis over a 10 week period. Immunologic parameters evaluated include gross cecal morphology, histopathology, mucosal immunohistochemistry, isotype of the antigen-specific serum antibody (ELISA), and antigen-induced Th1:Th2 cytokine responses. Results: Results indicate that H. bilis colonized gnotobiotic mice demonstrate diverse antigen-specific immune responses to their resident flora in comparison to gnotobiotic C3H mice having a defined flora alone. The immunologic responses of the gnotobiotic C3H mice colonized with H. bilis developed rapidly, generally persisted over the 10 week study and demonstrated a mixed Th1:Th2 phenotype. Although marked histologic lesions were not observed in H. bilis-colonized mice, alterations in mucosal B220 and CD4⁺ T cell and CD8⁺ T cell populations indicated that local immunity was disturbed. Conclusions: Gnotobiotic animals provide a useful tool for evaluation of the provocative potential of intestinal bacteria in the pathogenesis of intestinal inflammation. These studies support a potential pathogenic role for H. bilis in perturbing the normal resident microflora.
and inducing antigen-specific immune responses against multiple resident bacterial species. Our model suggests that changes in the homeostatic balance of the intestinal microbiota (following colonization with *H. bilis*) and the resulting host response may initiate mucosal inflammation, particularly in a setting where natural tolerance to the bacterium does not exist.
Introduction

The mammalian gastrointestinal (GI) tract harbors a complex microbial population which is remarkably stable throughout life and resists colonization by pathogenic bacteria.[1] This ecosystem is vast, both quantitatively and in terms of diversity, with the presence of at least 400-500 different species.[2] Most gut bacteria are commensals that coexist with the host in a mutualistic relationship. Several studies have demonstrated a beneficial role for the GI microflora in the provision of essential nutrients (e.g., vitamin K, short-chain fatty acids), the development of intestinal epithelium, vasculature, and lymphoid tissue, and the contribution to colonization resistance which protects the host from pathogenic infections.[3][4]

In contrast, altered composition and functional activities of the luminal microflora have been linked to the pathogenesis of chronic enterocolitis, such as inflammatory bowel disease (IBD). A number of animal models of human IBD provide compelling evidence for bacterial flora driving an inflammatory process. For example, both IL-2" [5] and IL-10" [6] mice spontaneously develop IBD when housed under conventional conditions, but there is no evidence of colitis when animals are maintained under germ-free conditions.[7] Adoptive transfer of CD45RB\textsuperscript{hi} T cells mediates less severe wasting disease in immunodeficient mice harboring a reduced intestinal flora [8]; furthermore, T-cell mediated gut inflammation can be transferred with effector T cells against enteric bacteria.[8][9] It appears that a constant bacterial stimulus is needed for the induction and perpetuation of inflammation [10], and that resident bacteria have different capacities to induce mucosal inflammation.[11][12][13] These accumulated observations incriminate intestinal bacteria in the initiation and perpetuation of chronic intestinal inflammation, although the critical bacterial species or
antigen(s) remain undetermined. It is likely that select components of the normal microflora and the antigen-specific immune responses that they evoke are crucial for the development of colitis.

Infection with *Helicobacter* species has been associated with the development of mucosal inflammation and IBD in several mouse models.[14][15][16][17][18][19] While some studies indicate that *Helicobacter* species do not induce IBD in germ-free IL-10^−/−^ mice or worsen IBD in IL-10^−/−^ mice that have been reconstituted with specific pathogen free (SPF) flora [7][20], others have shown that *Helicobacter* can be an important factor contributing to murine colitis in an SPF environment.[21][22] It is clear that the consensus opinion regarding the role of *Helicobacter* as a potential pathogen or its ability to interact with commensal luminal bacteria and induce intestinal inflammation is confounded by the presence of a complex colonic microbiota. The aim of this study was to determine if introduction of a novel bacterial species, *Helicobacter bilis*, into the microflora would perturb the homeostatic balance (e.g., immunologically) between the host and its resident flora.

**Materials and Methods**

**Animals**

Four-to-8 week old male and female C3H/HeN mice populated with a defined flora (e.g., altered Schaedler flora [ASF] comprised of 8 bacterial species) were obtained from Taconic Farms (Albany, NY). All mice were certified free of *Helicobacter* spp. by the vendor and re-tested on-site prior to study enrollment. Cohorts of mice were bred and maintained within the murine gnotobiotic facility at the College of Veterinary Medicine, Iowa State University (ISU). Animals were housed in Trexler plastic isolators using standard
gnotobiotic techniques. Mice were fed ad libitum an irradiated diet (e.g., Harlan-Teklad) and autoclaved water. All animal procedures were approved by the ISU Animal Care and Use Committee.

**Experimental Design**

Gnotobiotic C3H mice were assigned to one of two study groups: (1) defined flora alone receiving sham inoculations or (2) defined flora mice colonized with *H. bilis*. Representative experimental and control mice (6-12 mice per time point) were euthanized by CO₂ asphyxiation on days 21, 42, or 60 post-infection. Tissue samples were processed for evaluation of gross lesions, bacteriology, histopathology, immunohistochemistry, serum antibody levels, concentration of cytokines, and polymerase chain reaction (PCR) analysis for bacterial colonization.

**Infection with Helicobacter bilis**

An *H. bilis* isolate (ATCC strain 51630) was kindly provided by Dr Nancy Lynch (College of Medicine, University of Iowa). Organisms were streaked onto Columbia blood agar plates supplemented with 5% horse serum and grown under microaerophilic conditions (80% N₂, 10% H₂, and 10% CO₂) and kept at 37° C. Bacteria were collected from 3-5 plates and suspended into tryptic soy broth on the day of inoculation. Prior to inoculation, organisms were collected under sterile conditions and examined for their purity, morphology, and motility by dark phase microscopy. Samples were confirmed to be urease positive. Organisms were then suspended in broth to an approximate concentration of 10⁸-10⁹ CFU/ml. Gnotobiotic mice were orally infected with a bacterial inoculum (0.3 ml) of *H. bilis* administered every other day for three doses. Confirmation of *H. bilis* infection post-inoculum was made by PCR evaluation of feces or cecal contents and bacteriological culture.
**Helicobacter bilis and ASF-Specific PCR**

Fecal samples or cecal contents were analyzed for the presence of *Helicobacter* spp and *H. bilis* as described previously.[23][24] For generic *Helicobacter* PCR, 5 µl of quantitated fecal DNA was used as template while for *H. bilis* PCR, 10 µl of quantitated fecal DNA was used as template for all samples. Confirmation of ASF colonization at the termination of the appropriate period was performed by PCR of fecal pellet DNA from defined flora mice using ASF-specific primers with minor modifications made to PCR conditions.[25] Primers and their sequences used are summarized in Table 1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Position of primer in ORF</th>
<th>Primer Sequences</th>
<th>Size of Band Produced</th>
<th>ASF Member</th>
</tr>
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<tbody>
<tr>
<td>50-F</td>
<td>980-1000</td>
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<td>360</td>
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<tr>
<td>50-R</td>
<td>87-1004</td>
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<td>49-F</td>
<td>73-90</td>
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<td>125</td>
<td>361</td>
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<tr>
<td>49-R</td>
<td>178-197</td>
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<td>792-811</td>
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<td>56-R</td>
<td>815-833</td>
<td>GTGCCGCTTACGCTTGATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer sequences for identification of ASF bacteria. Primer sequences for identification of ASF361 and ASF492 are different than previously published sequences.[25] Modifications to primer sequences for detection of ASF361 and ASF492 were based on optimized PCR conditions and product detection using a multiplex format as compared to bacterial DNA positive control. ASF 356 = Clostridium cluster XIV; ASF 360 = *Lactobacillus acidophilus*; ASF 361 = *Lactobacillus animalis*; ASF 457 = *Flexistipes* phylum; ASF 492 = *Eubacterium plexicaudatum*; ASF 500 = Low G + C-content spp.; ASF 502 = *Clostridium* cluster XIV; ASF 519 = *Bacteroides distasonis*. 


Preparation of H. bilis and Bacterial Antigens

*Helicobacter bilis* organisms were cultivated in Columbia broth, recovered, and the bacteria collected by centrifugation. The cells were washed with sterile PBS, the cell pellet was frozen, cells were lyophilized, and then stored at -20°C until used.

Each member of the schaedler flora (ASF 360, ASF 361, ASF 356, ASF 457, ASF 492, ASF 500, ASF 502, and ASF 519) was grown in anaerobically prepared medium and cultured at 37°C for five to seven days. Cells were grown in either reinforced clostridium medium (RCM) supplemented with 5 % calf serum (ASF 360, ASF 361, ASF 356, ASF 502, and ASF 519) or schaedler broth supplemented with 5 % sheep serum (ASF 457, ASF 492, and ASF 500). Because many of these organisms are extremely oxygen sensitive (EOS), all inoculations were performed in a Coy anaerobic chamber. Culture flasks were sealed in anaerobic jars and then placed in an incubator. As above, the cells were harvested from broth by centrifugation, washed in PBS, and lyophilized.

Bacterial antigens (e.g., *H. bilis* and all eight ASF strains) used for ELISA or for stimulation of lymphocyte cultures were prepared from the lyophilized cells. Briefly, cells were weighed and resuspended in buffer or tissue culture medium to a final concentration of 2 mg/mL (dry weight to volume). The cell suspension was then placed on ice and sonicated for 5 minutes to prepare whole cell sonicates (WCS). Sonication was performed using 30 second pulses to prevent the suspension from overheating (i.e., denaturing of the proteins). Cell disruption was monitored microscopically. Protein content was determined by BCA (Pierce Laboratories). For the preparation of antigen for in vitro stimulation of lymphocytes,
the WCS were sterilized by UV light. Additionally, sterility was confirmed by bacteriological methods (e.g., aerobic and anaerobic blood agar cultures). These methods were used to prepare all antigens used for these studies. In order to ensure that all studies were performed with the same lot of antigens, each of the bacterial species to be used was grown during the first six months of the project to provide sufficient antigenic mass to complete these experiments.

**Evaluation of Macroscopic Cecal Lesions**

Macroscopic cecal lesions were scored using previous published criteria.[26] In brief, ceca were evaluated macroscopically for atrophy, edema, and increased mucus in the ingesta, given a gross lesion score (0-3: 0 = normal, 3 = severe cecal lesion), and placed in 10% neutral buffered formalin for histopathologic evaluation.

**Histopathologic Evaluation of Murine Tissues**

Sections of cecum and proximal colon were routinely processed, embedded in paraffin, and sectioned at 5μm for microscopic examination. Cecal and intestinal mucosa were stained with hematoxylin and eosin (H&E) for assessment of inflammation. Tissue sections were coded to blind the pathologist (JH) to the infection status of the animal. The cecum and proximal colon from each mouse were scored on severity of mucosal epithelial damage, architectural/glandular alterations, and magnitude/character of lamina propria cellular infiltrate (Table 2).
Table 2. Scoring System for Histopathological Evaluation of Gastrointestinal Inflammation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Scoring Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal height</td>
<td>Ratio of crypt depth to width</td>
</tr>
<tr>
<td></td>
<td>Rising score from 1-5 (mild to severe hyperplasia)</td>
</tr>
<tr>
<td>LP immune cells</td>
<td>Lymphocytes/plasma cells are the expected population</td>
</tr>
<tr>
<td></td>
<td>Mixed inflammation = increased neutrophils</td>
</tr>
<tr>
<td>Erosions</td>
<td>Rising score from 0-5 (0 = no erosions; 5 = ulceration)</td>
</tr>
<tr>
<td>Inflammatory score</td>
<td>Denotes quantity of cellular infiltrates in the LP</td>
</tr>
<tr>
<td></td>
<td>Rising score from 1-5 (normal = severe inflammation with necrosis)</td>
</tr>
<tr>
<td>Edema score</td>
<td>Rising score from 1-5 (1 = mild edema; 5 = severe edema with architectural distortion)</td>
</tr>
<tr>
<td>Other</td>
<td>Submucosal lymphoid hyperplasia (present of absent)</td>
</tr>
</tbody>
</table>

Table 2. Scoring system used for the histopathological evaluation of gastrointestinal inflammation. LP = lamina propria.

Analysis of Antigen-Specific Serum Antibodies

Serum antibody levels in mice were determined by ELISA as previously described.[27] Briefly, 96-well plates were coated with sonicated whole cell lysates of each of the eight anaerobic bacteria comprising the ASF and *H. bilis*. Diluted serum (1:200) was added to each well and incubated at 4°C overnight. Then, alkaline phosphatase conjugated goat anti-mouse IgG1 or IgG2a was added and incubated for 1 hour at room temperature. Wells were developed using p-nitrophenyl phosphate at room temperature. Color changes were measured using an ELISA plate reader at 405 nm.

Immunohistochemical Analysis

Cecal tissue sections were placed in an enriched cryopreservative media (O.C.T.), snap frozen on dry ice, stored at -80°C, and later warmed to -20°C, sectioned at 6μm, and stained for identification of mucosal CD4⁺ and CD8⁺ T cells and B220 B⁺ cells. Endogenous
peroxidase was inhibited by incubating tissue in buffer containing 0.3% H₂O₂. Antibodies used for section staining included: biotinylated rat anti-mouse CD4 (clone GK 1.5; 1 µg/ml, Becton Dickenson); biotinylated rat anti-mouse CD8α (clone 53-6.7; 1 µg/ml, Southern Biotechnology); and biotinylated rat anti-mouse CD45R (B220) (clone RA3-6B2; 1 µg/ml, PharMingen). All monoclonal antibodies were diluted in 1% (vol/vol) FCS in PBS. Streptavidin-HRP (1:2000) was added and the tissues incubated for 30 minutes at room temperature. A diaminobenzidine chromogen was used to visualize stained cells and sections were lightly counterstained with hematoxylin. Mucosal cell populations were enumerated using an ocular grid. Cells were quantified by a single investigator (ZL) evaluating 5-10 randomly selected and optimally-oriented microscopic fields (160x or 400x) per section.

**Cytokine Analysis by Multiplexed Flow Cytometric Assay**

Lymphocytes isolated from the spleen and mesenteric lymph nodes were incubated at 2.5 x 10⁶ cells per ml with or without whole cell lysates of the ASF organisms and *H. bilis* for 72 hours. Cell cultures were treated with concanavalin A to evaluate the overall nature of the T cell response. Cell-free supernatants were harvested and analyzed for their concentration of Th1 (TNF-α, IFN-γ, IL-2, IL-12) and Th2 (IL-4, IL-10) cytokines using a multiplexed flow cytometric assay (The FlowMetric™ System, Luminex, Austin, TX). Cytokine concentrations were measured using a murine cytokine detection kit (Mouse Cytokine LINCOplex Kit, Linco Research, St. Charles, MO) specifically designed for use with the Luminex systems.
Statistical Methods

For immunohistochemical analysis, mucosal immune cell populations were expressed as mean cells per microscopic field ± standard error of the mean (SEM). Cells counts in the 2 murine groups (control versus *H. bilis*-infected) were then compared using a non-parametric analysis. Serum antibody data were evaluated in two ways. First, the ratio of absorbance recorded in *H. bilis*-infected mice to absorbance recorded in non-infected controls was determined at 3-, 6-, and 10-weeks PI. These antibody ratios were then expressed as the mean ratio OD (absorbance) ± standard deviation. Secondly, non-parametric analysis was performed on the means and SEM of the pooled optical density (absorbance) for all 8 ASF antigens at 3-, 6-, and 10 weeks PI. The IgG isotype responses to *H. bilis* antigen were assessed using identical analyses as per the pooled ASF antibody responses.

For analysis of cytokine concentrations, the study design had 4 factors: organ (2 levels), cytokine (5 levels), organism (8 levels) and time (3 and 4 levels). Comparisons among levels of factors (e.g., spleen versus lymph node) were performed by testing the difference in proportions of median responses (e.g., determined as the ratio of antigen-specific cytokine concentration to background [no stimulation] concentration) that were at least 4 times background level. The normal approximation to the binomial distribution was assessed as valid and was used as the inferential procedure.[28] These were planned comparisons (rather than post-hoc), so that it was not necessary to adjust the *P*-values to control for type I error inflation.[29] A *P*-value of < 0.05 was considered statistically significant for all inferences.
Results

Colonization with *H. bilis* and ASF Bacteria

Mice inoculated with *H. bilis* became positive by fecal PCR 12-14 days post-infection and remained so for the 10 week study period (Figure 1). Both *Helicobacter* genus and *H. bilis*-specific primers consistently detected colonization. In some instances, culture of cecal contents at necropsy was also performed to confirm colonization with *Helicobacter* spp. Uninfected control animals remained *Helicobacter* fecal PCR and bacteriologically negative. Specific PCR products were reliably obtained by amplification of feces or cecal contents obtained at necropsy from defined flora mice using the ASF-specific primers in a multiplex format. Consistent with recent observations [25], detection of ASF member 360 from cecal contents of different mice at different time points was variable.

![Agarose gel](image)

**Figure 1.** Photograph of an agarose gel depicting PCR analysis of murine fecal samples for the presence of *H. bilis*. C3H/HeNTac mice bearing a defined bacterial flora were colonized six weeks prior to sample collection with *H. bilis*. Fecal samples were collected and extracted using the QIAamp DNA Stool kit (Qiagen) to recover bacterial DNA. Using specific PCR primers for *H. bilis*, fecal samples from 11 separate mice were assayed for the presence of *H. bilis*. Sample 1, DNA from pure culture of *H. bilis*; Samples 2-10, fecal samples from gnotobiotic mice orally inoculated with *H. bilis* six weeks earlier; Samples 11-12, fecal samples from control gnotobiotic mice sham inoculated with sterile culture media. Outside lanes contain DNA size markers.
Clinical, Gross, and Histopathologic Findings Observed in *H. bilis*-Infected Defined Flora Mice

Over the 10 weeks of the study, wasting disease and overt diarrhea were not observed in *H. bilis* infected mice. Similarly, there was no significant weight change in *H. bilis* infected mice as compared to non-infected controls (data not shown). Significant differences in cecal morphology subsequent to *H. bilis* colonization were not observed at necropsy; however, infected ceca were uniformly smaller with regional mesenteric lymphadenopathy frequently present in 6- and 10-week infected mice as compared to non-infected controls.

Microscopic changes of mild inflammation were observed in cecal tissue recovered from 3- and 6-week *H. bilis* infected mice. Histopathologic findings included increased cellular infiltrates, diffuse epithelial erosions, and submucosal edema. The cellular infiltrate in these specimens was confined to the lamina propria and consisted primarily of lymphocytes and plasma cells. While an increase in inflammation (e.g., increased mucosal cellularity, mucosal hyperplasia, submucosal edema, lymphoid hyperplasia [Figure 2]) and histology scores was noted for cecal tissues from 10-week infected mice, these changes were not significant.
Figure 2. Histological changes in the cecal mucosa of defined flora mice after infection with *H. bilis*. (A) Controls – no infection; (B) Mice infected with *H. bilis* for 6 weeks – mild typhlocolitis is evident and is characterized by the presence of submucosal edema, mild infiltration of the lamina propria (LP) with mononuclear cells and mild crypt hyperplasia; (C) Mice infected with *H. bilis* for 6 weeks – moderate typhlocolitis as evidenced by marked mononuclear and neutrophilic infiltrates in the LP accompanied by moderate crypt hyperplasia. All images at 100X.

Antigen-Specific IgG1/IgG2a Antibody Responses Subsequent to *H.bilis* Colonization

*H.bilis*-infected gnotobiotic mice demonstrated diverse antigen-specific immune responses to their resident flora in comparison to non-infected mice (Figure 3A). Antibody responses developed quickly, persisted over the 10-week study, and they demonstrated a mixed Th1:Th2 phenotype. At all time points evaluated, pooled IgG1 and IgG2a responses to ASF antigens were significantly (*P*<0.05) increased in infected mice as compared to non-infected controls (Figure 3B). Furthermore, the IgG1 response to pooled ASF antigens at 6-weeks PI was significantly (*P*<0.05) increased as compared to the IgG1 levels seen in 10-week infected mice. It is noteworthy that IgG responses to several ASF members including ASF 457 (increased IgG2a at 10-weeks PI), ASF 500 (increased IgG2a at 10-weeks PI), ASF 502 (increased IgG1 at 3-weeks PI), and ASF 519 (increased IgG2a at 10-weeks PI) were significantly (*P*<0.05) increased as compared to the antibody response to other ASF members. The IgG1 and IgG2a responses to *H.bilis* were significantly (*P*<0.05) increased at
6-weeks PI when compared to antibody responses observed in mice 3- and 10-week PI.

Serum collected from control DF mice did not demonstrate any antibody response to *H. bilis* antigens (Figure 4) at any time point (data not shown).

**Figure 3A.** Serum immunoglobulin responses from *H. bilis*-infected defined flora (DF) mice and non-infected DF mice. Immunoglobulin isotypes (IgG1 and IgG2a) to bacterial antigens of the altered schaedler flora (ASF) at 3-, 6-, and 10-weeks post-*H. bilis* infection are shown. *H. bilis*-infected DF mice produce a broad immunoglobulin response to numerous ASF antigens which generally peaks at 3 to 6 weeks post-infection. Numbers reflect means and standard deviations of the ratio of optical density (e.g., ratio OD calculated as the ratio absorbance in *H. bilis*-infected mice to absorbance in non-infected mice). Each mean value comprises triplicate wells of individual mouse serum from 4-8 mice per group averaged over 3 independent experiments. ASF 356 = Clostridium cluster XIV; ASF 360 = *Lactobacillus acidophilus*; ASF 361 = *Lactobacillus animalis*; ASF 457 = *Flexistipes* phylum; ASF 492 = *Eubacterium plexicaudatum*; ASF 500 = Low G + C-content spp.; ASF 502 = *Clostridium* cluster XIV; ASF 519 = *Bacteroides distasonis*. 
**Figure 3B.** Pooled serum immunoglobulin levels from *H. bilis*-infected defined flora (DF) mice and non-infected (control) DF mice. Serum immunoglobulin responses (IgG1 and IgG2a) to bacterial antigens of the altered schaedler flora (ASF) at 3-, 6- and 10-weeks post-*H. bilis* infection were determined by ELISA as described in materials and methods. Numbers reflect the means and SEM of the pooled optical density (absorbance) for all 8 ASF antigens averaged over 3 independent experiments.

* P<0.05 for *H. bilis*-infected mice versus control (non-infected) mice.
† P<0.05 for *H. bilis*-infected mice at 6 weeks versus *H. bilis*-infected mice at 10 weeks.
**Figure 4.** Pooled serum IgG isotype responses from *H. bilis*-infected defined flora mice. Absorbance determined at 3-, 6-, and 10-weeks post-infection (PI) as described in materials and methods. Numbers reflect the means and SEM of the pooled optical density (absorbance) for *H. bilis* IgG1 and IgG2a averaged over 3 independent experiments. * P<0.05 for IgG1 at 6 weeks-PI versus 3- and 10-weeks PI. † P<0.05 for IgG2a at 6 weeks-PI versus 3- and 10-weeks PI.

**Alterations in Mucosal B and T Lymphocyte Populations in *H. bilis*-Infected Mice**

Colonization of defined flora C3H mice with *H. bilis* was associated with altered mucosal populations of B and T lymphocytes. As compared to non-infected control mice, ocular morphometry revealed that CD4⁺ T cells were the most numerous mucosal immune cell within the lamina propria in infected mice followed by lesser numbers of CD8α⁺ T cells and B220 cells. For each cell type evaluated, there appeared to be a similar trend with mean cell numbers peaking at 3 weeks then declining towards the number of cells detected at time 0 (baseline) by week 10 (Figure 5).
Figure 5. Alterations in mucosal T and B lymphocyte populations from *H. bilis*-infected defined flora (DF) mice (red bars) and non-infected DF mice (green bars). Mucosal cellularity of CD4⁺ T cells, CD8α⁺ T cells and B220 cells is increased at 3-, 6-, and 10-weeks post-infection in *H. bilis*-infected mice compared with non-infected DF mice. Two columns of data are shown. **Left column** – mean cells per microscopic field and standard deviations derived from pooled tissues of 6-8 mice per group. **Right column** – representative immunohistochemical staining of cecal tissues using monoclonal antibodies for CD4 (top), CD8α (middle), and B220 (bottom).

*P<0.05 for *H. bilis*-infected versus non-infected for each cell type.
Antigen-Specific Cytokine Responses Obtained from Lymphocyte Cultures

Supernatants derived from antigen stimulated lymphocytes derived from *H. bilis*-infected defined flora mice showed elevated levels (pg/ml) of IFN-γ, TNF-α, IL-6, IL-10, and IL-12 but marginally detectable levels of IL-2 and IL-4. Therefore, subsequent analysis of IL-2 and IL-4 cytokines was not performed. Overall, more numerous and robust (at least four-fold) responses to ASF members were observed from antigen-stimulated splenic lymphocyte cultures (Table 3). In contrast, mesenteric lymph node supernatants produced lesser quantities of each cytokine at all time points with the exception of IL-6. Evaluation of these immunologically compartmentalized responses demonstrated that the overall splenic response (e.g., concentration ratios for IFN-γ, TNF-α, IL-6, and IL-10 at 3-, 6-, and 10-weeks post-infection and for IL-12 at 3- and 6-weeks post-infection) significantly (*P*<0.05) exceeded the cytokine response derived from mesenteric lymph node cultures (Table 4).

Significantly (*P*<0.05) greater overall cytokine responses were induced by stimulation with ASF members 360, 500, 502, and 519 in comparison to responses of stimulated lymphocytes derived from the mesenteric lymph node. A comparison of individual cytokine responses between immune compartments showed that the concentration derived from splenic lymphocyte cultures for each cytokine was significantly (*P*<0.05) greater than the ratio produced by the same cytokine derived from mesenteric lymph node cultures (e.g., splenic IFN-γ > mesenteric lymph node IFN-γ, etc.).
<table>
<thead>
<tr>
<th>Mesenteric Lymph Node Culture</th>
<th>Splenic Culture</th>
</tr>
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<tbody>
<tr>
<td>Reactive *ASF Bacteria</td>
<td>Reactive *ASF Bacteria</td>
</tr>
<tr>
<td>356 360 361 457 492 500 502 519</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>X</td>
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</tr>
<tr>
<td>3w-PI</td>
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<tr>
<td>X X X X X X X X X X</td>
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</table>

Table 3. Increased concentrations of cytokines in mesenteric lymph node (MLN) and splenic (SPL) supernatants derived from altered schaedler flora (ASF) bacteria stimulated lymphocytes in *H. bilis*-infected defined flora (DF) mice and non-infected DF mice. Lymphocytes were cultured for 72 hours, then supernatants were harvested and surveyed via multiplexed flow cytometric assay. *Reactive ASF bacteria are denoted by an X, and comprise those bacterial members which evoked at least a four-fold increase in median cytokine concentration (determined as the ratio of antigen-specific cytokine concentration to background [no stimulation] concentration) subsequent to stimulation. Note the apparent compartmentalized responses between MLN and SPL cultures. ASF 356 = Clostridium cluster XIV; ASF 360 = *Lactobacillus acidophilus*; ASF 361 = *Lactobacillus animalis*; ASF 457 = *Flexistipes* phylum; ASF 492 = *Eubacterium plexicaudatum*; ASF 500 = Low G+ C-content spp.; ASF 502 = *Clostridium* cluster XIV; ASF 519 = *Bacteroides distasonis*. 
Table 4. Summary data of cytokine analysis in gnotobiotic mice. See methods section for explanation of statistical analyses performed. *Analyses reflect direct comparison of median cytokine concentrations derived from splenic (SPL) and mesenteric lymph node (MLN) cultures. For all comparisons, a P-value < 0.05 is considered statistically significant. ASF 356 = Clostridium cluster XIV; ASF 360 = Lactobacillus acidophilus; ASF 361 = Lactobacillus animalis; ASF 457 = Flexistipes phylum; ASF 492 = Eubacterium plexicaudatum; ASF 500 = Low G+ C-content spp.; ASF 502 = Clostridium cluster XIV; ASF 519 = Bacteroides distasonis.

a Comparison of all cytokines (e.g., IFN-γ, TNF-α, IL-6, IL-10, and IL-12) across all time points (e.g., time 0, 3-, 6-, and 10-weeks post-\textit{H. bilis} infection for all cytokines but IL-12 which was evaluated only at 0, 3-, and 6-weeks post-\textit{H. bilis} infection).

b Only supernatant samples at time 0, 3-, and 6-weeks post-\textit{H. bilis} infection were available for analysis.


Discussion

Determining the relative contributions of host responses to enteric bacteria which modulate intestinal inflammation is problematic due to the presence of a complex microbiota. To assess which subsets of resident bacteria are most responsible for induction of experimental gastrointestinal inflammation, gnotobiotic studies employing a defined flora host colonized with a single (e.g., monoassociated) bacterium or combination of a limited number of bacteria species have been performed. Reconstitution studies in gnotobiotic HLA-B27 transgenic (TG) rats containing six different obligate and facultative intestinal anaerobic bacteria, including *Bacteroides vulgatus*, develop much more active colitis and gastritis than littermates colonized with the same selected bacteria without *B. vulgatus*. Furthermore, HLA-B27 TG rats colonized with SPF bacteria had more inflammation than gnotobiotic rats colonized with the defined flora including *B. vulgatus*. In subsequent monoassociation studies, it has also been shown that *B. vulgatus* but not *E. coli* induced colitis of equal severity to that observed in gnotobiotic TG rats. *Helicobacter* species may or may not be associated with gastrointestinal inflammation in different animal models depending upon the composition of the resident flora and host susceptibility. These accumulated results suggest that a subset of luminal bacteria (especially anaerobes) have differing abilities to contribute to the onset of colitis, that introduction of a novel bacterium perturbs the local microbial ecology thereby provoking host immune responses, and that this ecological and/or immunological perturbation provides the constant antigenic stimulus for immune-mediated colonic inflammation.

Other studies performed using a variety of experimental models have shown that the resident microflora plays an important role in the pathogenesis of
IBD. In most of these models, Th1 immune responses are directed toward commensal intestinal bacteria and contribute to gut inflammation. Salient examples of these phenomena include colitis which may be caused by the transfer of bacterial antigen-specific CD4+ T cells from spontaneously colitic C3H/HeJ Bir mice [9]; the observation that monoassociated IL-10-/- mice develop antigen-specific immune responses to colitis-producing bacteria (e.g., E. coli, Enterococcus faecalis) but not to bacteria incapable of producing disease [40]; the finding that CD4+ T cells derived from C3H/HeJBir mice demonstrate strong antibody (e.g., IgG2a) reactivity to certain antigens derived from the resident enteric flora (e.g., Enterococcus spp. and Enterobacteriaceae) but not to epithelial cells or to food antigens [41]; and observations by Onderdonk et al that show that high concentrations of E. coli and Enterococcus species are associated with severe colitis in HLA-B27 TG rats. [42] While it is clear that luminal bacteria have important but complex roles in initiating and sustaining intestinal inflammation, the contribution of antigen-specific host responses directed against resident flora in this process has not been critically evaluated.

We initially investigated host immune reactivity to the resident microflora in conventionalized C3H mice infected with the spirochete, Brachyspira hyodysenteriae. Previous experiments have indicated that infected mice develop a polarized Th1 response characterized by prominent IgG2a production, increased IFN-γ secretion, and severe typhlocolitis (MJ Wannemuehler, unpublished data). The resident microflora is a important cofactor for disease development in this model as the use of antibiotic therapy to selectively deplete members of the microflora (e.g., gram-negative anaerobes) abolishes the ability of the spirochete to induce colitis. [45] In subsequent studies, we have confirmed the presence of Helicobacter spp. in inflamed ceca suggesting that these
bacteria potentially contribute to intestinal inflammation in infected mice.[46] To further explore the pathogenesis of microbial-induced colitis, we infected gnotobiotic C3H mice (harborin an ASF flora) with *H. bilis* and evaluated antigen-specific immune responses to the resident flora. We found that the introduction of *H. bilis* into defined flora mice evoked diverse host responses to commensal ASF members and that this perturbation may “trigger” the development of colitis.

*Helicobacter* spp. have been incriminated as provocateurs of microbial-induced colitis in a number of animal models. In separate experiments, *H. hepaticus* induced chronic colitis in SPF-reared IL-10−/− mice[21] and caused significant mucosal inflammation in the cecum and colon of TCR-αβ mutant mice.[15] Burich et al observed that both *H. bilis* and *H. hepaticus* may cause histologic lesions of EBD of varying severity in genetically susceptible hosts.[22] The monoassociation of SCID mice with *H. muridarum* provokes the development of IBD; however, these mice did not develop wasting disease or colitis.[19] Still other models suggest a less pathogenic role for *Helicobacter* spp. and have shown that *Helicobacter* does not induce IBD in germ-free IL-10−/− mice[20] or potentiate IBD in germfree IL-10−/− mice reconstituted with a SPF flora.[7] Also, *H. hepaticus* infection delays the development of IBD in mdrlα−/− mice.[47] We and others[22] suggest that the contrasting observations from some studies are due to variability in genetic susceptibility to IBD and to different compositions of intestinal flora in these animal models. The use of an immunocompetent gnotobiotic mouse which harbors a defined flora with a limited number of bacterial species (which are stable from mouse to mouse), such as the model used in the present study, represents a unique opportunity for evaluating the specificity of the resident flora in the initiation of intestinal inflammation.[25] Additionally, evaluation of host
responses in mice having a functionally normal immune system is more realistic to the human condition, where IBD predominantly effects immunologically-competent patients.

Defined flora mice infected with *H. bilis* developed only mild, histologically-evident typhlocolitis after chronic colonization. Significant wasting disease with watery diarrhea was not observed; however, many mice were noted to have pasty, mucoid feces at necropsy. Gross lesions of cecal involution and mesenteric lymphadenopathy were common in 6- and 10-week infected mice in comparison to non-infected gnotobiotic mice. Histologic lesions of mild typhlocolitis were characterized by increased mucosal infiltrates, submucosal edema, and increased inflammatory scores which worsened over time. Additionally, both the quantity and character of the lamina proprial infiltrate changed (e.g., from a mononuclear cell infiltrate to that containing increased neutrophils) in tissues evaluated from 10-week *H. bilis*-infected mice. Our findings of mild colitis in mice colonized with *H. bilis* have been reported by others using Ragl−/− mice, that lack both B and T lymphocytes.[22]

An unexpected finding in this study was the balanced Th1:Th2 profile of host immune responses following colonization with *H. bilis*. In most instances, these responses were persistent with regards to cytokine production and exhibited distinct patterns of compartmentalization between the spleen and mesenteric lymph node. Gnotobiotic mice infected with *H. bilis* developed a dominant serum IgG2a antibody response at 6 weeks PI (e.g., Th1 profile) as previously observed in other animal models infected with *Helicobacter* spp.[21][48][49][50] Antibody responses to ASF members for both immunoglobulin isotypes followed a similar pattern of peaking at 6 weeks post-infection. The observed IgG1 (e.g., Th2-like) response to select ASF bacteria following *H. bilis* colonization was unexpected. Our hypothesis for this observation is that this Th2 immune bias develops
following the introduction of *H. bilis* into the gut because this alters or perturbs the local microbial ecology, thereby influencing the immunological response of the host to non-pathogenic luminal bacteria.

We observed disturbances in local immunity (e.g., alterations in mucosal B and T lymphocyte populations) of *H. bilis*-infected mice that broadly correlated with peak antibody responses. Immunohistochemical studies performed on cecal tissues of *H. bilis*-infected mice showed increases in CD$^4^+$ T cells, CD$^8^+$ T cells, and B220$^+$ cells which then decreased to baseline values by week 10 of infection. The phenotype (e.g., helper or regulatory T cell) and antigen specificity of the infiltrating CD$^4^+$ T cells was not determined; however, it is tempting to speculate that the increase was in response to conventional antigens of the bacterial flora as reported by others.[9][51]

Up-regulation of Th1 cytokines like IFN-γ and TNF-α has been noted in other bacterial models of IBD [52] and has been reported to characterize *Helicobacter*-induced disease.[21][22][49] Thus, it was not surprising to observe increases in these cytokines along with IL-6 in lymphocyte supernatants following antigenic stimulation. It was noteworthy that increased production of IL-12 was observed in lymphocyte cultures from both the lymph nodes and spleens recovered from mice colonized with *H. bilis*. The production of IL-12 from macrophages or dendritic cells may occur in response to diverse stimuli including live bacteria, bacterial products such as LPS and lipotechoic acid, and/or prokaryotic DNA.[53][54][55][56] The results of this study suggest that resident luminal bacterial products may also stimulate IL-12 production. IL-12 induced by stimulation with microbial products has been associated with the development of autoimmune disease [39] and loss of tolerance to resident luminal bacteria in experimental colitis.[57] It is also possible that
increased IL-12 production caused excess production of pro-inflammatory IFN-γ, thereby, altering gut epithelial barrier integrity. While this contention would be histologically supported by the presence of overt inflammation, one explanation for the lack of mucosal inflammation in this model is that lesion severity was attenuated by the increased production of IL-10. Animal studies have shown that IL-10 may modulate inflammatory responses in IBD, presumably through regulatory T cell activity.[57][58] In contrast, Maggio-Price et al have shown that severe IBD may occur in conjunction with increased colonic IL-10 in mdr1a⁻ mice infected with H. hepaticus, and that delayed development of IBD is not necessarily accompanied by increased production of IL-10 in these same mice.[47] Future studies will more specifically address the role of IL-10 in modulation of typhlocolitis in H. bilis-infected gnotobiotic mice.

The up-regulated antigen-specific cytokine responses observed from splenic cultures demonstrate that compartmentalized host responses to the resident bacteria occur subsequent to H. bilis colonization. Furthermore, these data suggest that different members or subpopulations of resident bacteria have the potential to initiate host immune responses and contribute to the onset of colitis. Mechanisms by which H. bilis colonization evokes host immune responses in these mice are not fully known. As semi-quantitative PCR confirmed the presence of all ASF bacteria on necropsy, alterations in commensal members following H. bilis colonization seem unlikely. It is possible that quantitative differences in bacterial numbers or their location within the gut (e.g., luminal versus adherent – not measured in this study) following H. bilis infection might have influenced the intensity of immune responses to the individual ASF members.[7] Additionally, Helicobacter spp. may initiate the development of intestinal inflammation through diverse mechanisms including the
elaboration of specific toxins (e.g., cytolethal distending toxins) [59], IFN-γ-mediated compromise of intestinal epithelial barrier function [47][60], the recruitment of commensal-specific CD4+ T cells to the colonic lamina propria [9], LPS-mediated activation of toll-like receptors found on dendritic cells and macrophages which trigger the innate immune system [22], and/or altered epithelial cell gene expression. Whether some or all of these mechanisms are responsible for the initiation of intestinal inflammation in immunocompetent C3H mice bearing a defined flora mice following colonization with *H. bilis* remains to be determined.

These results indicate (suggest) that perturbation of the microbial ecology, following the introduction of a novel bacterial species such as *H. bilis*, may predispose or set the stage for the development of colitis in gnotobiotic mice. It would appear that normal luminal bacteria play an important role in the initiation of host immune responses, that these responses are of mixed Th1:Th2 phenotype, that these responses are compartmentalized in their magnitude, and that further studies are warranted. Lastly, some commensal bacteria may exert a more dominant role than others in the induction of colitis, particularly in a setting where natural tolerance to the introduced bacterium does not exist.[22]
References


61:130-143.
CHAPTER 4. GENERAL CONCLUSIONS

To better understand how bacteria "trigger" intestinal inflammation in a susceptible host, we have developed a novel mouse model of bacterial-induced colitis. In a series of subsequent experiments, we used this model to investigate host-microbial interactions contributing to the development of typhlocolitis in gnotobiotic mice. This model would appear to have several advantages over other models of intestinal inflammation including: (a) the initiating etiologic agent (B. hyodysenteriae) is known; (b) mice having a defined flora with a limited number of bacterial species (which are consistent from mouse to mouse) is needed to develop gut inflammation; (c) this model provides reproducible typhlocolitis quickly and consistently allowing chronological studies of both acute and chronic inflammation to be performed; and (d) immunocompetent mice are used to assess the host's immune response following onset of colitis.

While immune responses of the gnotobiotic mice of in our preliminary studies were of mixed Th1/Th2 phenotype, the original pilot data (e.g., experiment 1 in the dual-infection study [paper 2]) did not establish whether these responses were persistent over time or the nature of the microbial populations associated with them. The H. bilis temporal studies (paper 1) did address the kinetics of the host immune responses to the ASF bacteria over time in a systematic fashion. These additional experiments revealed that host responses persist ad a mixed Th1:Th2 expression over the 10-week study period, that these responses are compartmentalized (e.g., spleen versus mesenteric lymph node) in their magnitude, and that some commensal bacteria may exert a more dominant role than others in the induction of host responses modulating the development of colitis. These accumulated data clearly indicate that future studies evaluating host responses following introduction of a novel
bacterium in defined flora mice are warranted. In particular, we would like to more critically evaluate sub-populations of the ASF and the relative contributions they make with regards to generation of host immune responses.

FUTURE STUDIES OVER THE NEXT 2 YEARS

Specific Aim. Evaluate the characteristics of the resident flora that are critical in eliciting immune responses in experimental colitis.

Rationale: These proposed experiments will examine host-microbial interactions which test the hypothesis that specific resident bacteria are important in triggering immune responses. Based on our preliminary data, we have noted that select ASF bacteria in gnotobiotic mice tend to be more or less immunogenic as previously measured. However, which critical “mix” of flora is most important at evoking immune responsiveness has not been demonstrated. Selective bacterial reconstitution studies in germfree mice will be performed to evaluate the resultant immune response. Characterization of the identity and location of ASF bacteria contributing to antigen-specific immunity will be performed by fluorescent in situ hybridization (FISH).

Experimental Design

Derivation of germ free mouse groups.

Three to six week old germfree (GF) C3H mice will be obtained from Taconic Farms. Foundation stock for this strain is cesarean-derived with expansion stock maintained under germfree conditions. All mice will be certified free of bacterial species, including Helicobacter spp., by the vendor and retested on site before each experiment. Animals will
be strictly housed in a germfree environment in gnotobiotic isolators while at our facility. The GF mice will be assigned to one of 4 treatment groups based on \textit{H. bilis} and \textit{B. hyodysenteriae} infection status: Group 1 (control) - entirely GF with no bacterial infection; Group 2 - infected with \textit{B. hyodysenteriae} alone; Group 3 - infected with \textit{H. bilis} alone; Group 4 - infected with \textit{H. bilis} and \textit{B. hyodysenteriae}. Following bacterial reconstitution experiments (described below), representative control and experimental mice will be euthanized on day 75 PI and samples will be taken for gross cecal morphology, histopathology, immunohistochemistry, bacteriology, serum antibody levels, B cell ELISPOT, and levels of cytokines.

**Bacterial reconstitution studies.**

The 8 ASF species will be equally divided into 2 types of organisms based on their ability to evoke antigen-specific immune responses as measured in our preliminary studies. **Type A** organisms will consist of those defined flora bacteria which were generally observed to be more immunogenic - ASF members 356, 361, 457, and 492. **Type B** organisms will consist of those defined flora bacteria which were generally observed to be less immunogenic - ASF members 360, 500, 502 and 519. Earlier work has established the need to introduce a \textit{Lactobacillus} spp. (e.g., ASF 360 or 361) first, to facilitate colonization by other more oxygen-sensitive ASF members. Presumably, these \textit{Lactobacilli} lower the luminal redox potential favoring growth conditions for EOS bacterial species. The 4 GF murine groups will then be colonized by both Type A and Type B defined bacterial species in separate experiments. We will next compare the effects of Type A (more immunogenic) bacteria and Type B (less immunogenic) bacteria on induction of Th1 and Th2 immune responses in individual gnotobiotic groups.
Establishment of H. bilis and B. hyodysenteriae infections in GF mice.

The establishment of *H. bilis* and *B. hyodysenteriae* infections in GF mice will be performed as previously described. Dual infected GF mice will be first orally infected with *H. bilis*, followed 21 days later by oral administration with $2 \times 10^8$ *B. hyodysenteriae* organisms.

Assessment of immunopathologic parameters.

Following euthanasia, samples will be taken for assessment of gross cecal lesions, histopathology, immunohistochemistry, bacteriology, analysis of antigen-specific serum antibodies, B cell ELISPOT, and cytokine analysis. These results will be compared between Type A and Type B ASF members, with Type A members predicted to elicit greater Th2 immune responses, especially in dual infected GF mice.

Identification of ASF species using a fluorescent in situ hybridization technique.

Although previous data indicate that immune responses to select ASF members occur, confirmation of their mucosal location (e.g. intraluminal versus attaching) has not been performed. Furthermore, immune responses may be influenced by relative concentrations of select ASF. Fluorescent in situ hybridization (FISH) with probes targeting each ASF member will be used to quantitatively detect the presence and location of these bacteria. A limited number of probes are available targeting gut-associated bacteria. Prospective FISH probes will be validated experimentally for each bacterium in the ASF. If necessary, additional probes targeting 16S rRNA will be designed using Vector NTI software package (InfoMax, Bethesda, MD) with GenBank and RDP databases. Specificity of probes will be determined experimentally. Tissues will be formaldehyde-fixed at time of necropsy
and stored at -20°C until hybridized. One to three probes, with different fluorescent labels, will be hybridized to a sample in a single operation following standard FISH protocols. Hybridized samples will be visualized and images captured using a fluorescence microscope fitted with a digital camera and imaging software. These studies will be performed under the guidance of Dr. Cherie Ziemer’s laboratory at the USDA.

**Anticipated Results**

It is our intent to use these gnotobiotic GF mice colonized with a subset of the ASF organisms to identify which collection of ASF bacteria is most important in driving host immune responses. It is our expectation that infection of GF mice with Type B ASF bacteria (e.g., less immunogenic species) will evoke less immunologic responses as compared to Type A ASF bacteria which tend to be more immunogenic. Based on our previous data, we surmise that immune responses to ASF bacteria following *H. bilis* infection will be predominantly Th2-like, while immune responses to ASF bacteria following *B. hyodysenteriae* infection will be characteristically Th1-like. Thus, GF mice infected with *H. bilis* should have no gross and histologic lesions, similar to non-infected GF controls. In contrast, GF mice infected with *B. hyodysenteriae* will have significant mucosal inflammation as a consequence of Th1-mediated responses to their ASF bacteria. Lastly, dual-infected GF mice should have intestinal inflammation of intermediate severity between these two extremes as Th2 responses elicited by the presence of *H. bilis* with the ASF flora modulate (down-regulate) the Th1-driven immunity to *B. hyodysenteriae*. 
Potential Difficulties, Limitations, and Alternative Approaches

Arguably, the optimal time point for evaluation of C3H GF mice has not been established. The choice of PI day 75 for necropsy is arbitrarily based on very limited pilot studies in GF BALB/c mice which failed to demonstrate consistent intestinal lesions until 60-70 days following B. hyodysenteriae infection. It is quite possible that our expected results may vary since we will be evaluating the gnotobiotic mice later in disease progression and we will be evaluating subsets of the ASF bacteria rather than the total ASF flora. It is also possible that some of the ASF organisms may not induce detectable immune responses regardless of the time that evaluation occurs.

There are also some potential pitfalls regarding FISH analysis of the flora. Preparation of gut wall samples will require some adaptation of other established procedures. Only 2 of the ASF strains have been typed as species which may require some 16S rDNA sequencing to determine where they fall prior to application or design of probes. This may need to be done for all ASF members in order to confirm 16S rDNA phylogeny for correctly determining target sequences (even for using available probes but especially for designing probes). If the Clostridium and Lactobacillus strains are too closely related it may be difficult or even impossible to develop probes specific enough to differentiate among them.

FUTURE STUDIES ANTICIPATED OVER THE NEXT 3-5 YEARS

Experiments during this time frame will be in line with the long term goals of our laboratory and will evolve, to some degree, on results generated from the germ-free mouse studies. We have previously shown that the introduction of H. bilis into gnotobiotic mice which are subsequently co-infected with B. hyodysenteriae alters host immune responses to
bacterial antigens modulating the severity of colitis. This response might be mediated through bacterial-epithelial cell interactions which in turn produce cytokines, chemokines, and other mediators which induce activation of the mucosal immune system. Several recent reports have demonstrated altered epithelial gene products involved in mucosal defense and inflammation associated with ubiquitous enteric bacteria. We hypothesize that Type A and Type B ASF bacteria will differ in their ability to modulate colitis in gnotobiotic mice, and that these differences will be associated with induction of different epithelial gene products which may be detected using microarray analysis and/or real time PCR. These procedures will be performed on intestinal specimens previously obtained from each group of gnotobiotic mice archived during completion of the germ-free studies. Gene expression in gnotobiotic mice will also be compared to that observed in conventional mice. This novel approach will allow the identification of known, and perhaps novel, gene products involved in mucosal defense against luminal microorganisms and their associated inflammatory response.

Mechanisms by which immune responses to the enteric microflora are controlled so that chronic inflammation does not occur in the gut have recently been demonstrated. Of interest, IL-10 deficient but not wild-type (WT) mice develop colitis after infection with Helicobacter hepaticus. Using a modification of the CD4/SCID IBD model, investigators induced colitis in H. hepaticus-infected recombination activation gene (RAG) knockout (KO) mice by the transfer of CD4+ T cells from infected, colitic, IL-10 KO mice. They subsequently analyzed the disease protective effects of the co-transfer of various CD4+ populations from WT mice. Results indicate that H. hepaticus infection induces a distinct population in WT mice of regulatory CD4^{+}CD25^{+} T cells which prevent bacterial-induced
colitis. The contribution of responses induced by antigens derived from the normal flora were not examined. These data were determined in mice colonized with a conventional, complex flora and whose immune responses were evaluated shortly after *H. hepaticus* infection.

We propose to further investigate the interaction between *H. bilis* and the normal flora in the development of a regulatory T (*T_{reg}*) cell response by studying changes in the responses of the *T_{reg}* cells recovered from the mucosa and MLN. It is unknown whether or not the *T_{reg}* cell response persists over time. For these studies, gnotobiotic mice will be infected with *H. bilis* and the cecal mucosa and MLN will be collected at various times after colonization. The CD45RB_{low} population of CD4+ T cells will be collected and stimulated with antigens from *H. bilis* or antigens of the ASF organisms. The CD4^{+}CD45RB_{low} *T_{reg}* cells will be enriched using magnetic bead separation (for CD4) and cell sorting (for the CD45RB_{low} population) using a flow cytometer. The recovered cells will be stimulated in vitro with specific antigens in the presence of IL-2 and culture supernatants will be analyzed for the production of IL-10 and/or TGF-β. These data will indicate whether *T_{reg}* cells are responsive to *H. bilis* antigens as well as to antigens derived from the normal flora. In addition, evaluating this response at several time points will provide evidence as to when this response is maximal and whether the response is differentially induced by antigens from the various members of defined flora.
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